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(54) Title: COMPOSITION CONTAINING FLAVONOIDS FOR TREATMENT BRAIN DISORDERS

(57) Abstract: The present invention relates to a pharmaceutical composition for use (or when in use) in the treatment of brain disorders, said composition comprising one or more flavonoids admixed with a pharmaceutically acceptable diluent, excipient or carrier. The invention also relates to a process for the preparation of said pharmaceutical composition. A further aspect of the invention relates to the use of one or more flavonoids in the preparation of a medicament for treating brain disorders, and to a method of treating a brain disorder, said method comprising administering to a subject in need of treatment a therapeutically effective amount of one or more flavonoids.

COMPOSITIONS CONTAINING FLAVONOIDS FOR TREATING BRAIN DISORDERS

The present invention relates to a pharmaceutical composition for treating brain disorders. More particularly, the invention relates to the treatment of brain cancers, and neurodegenerative disorders such as Parkinson's disease.

Current therapies for treating malignant gliomas, despite having the potential to prolong survival, are a usually a compromise between control of tumour growth and preservation of quality of life, rather than cure. A multidisciplinary regimen of de-bulking surgery in conjunction with radiotherapy and/or chemotherapy is frequently employed. The major biological feature of human brain tumours that precludes successful therapy is their propensity to invade the contiguous normal brain. Although intrinsic tumours of the brain seldom metastasise to distant sites, individual 'guerilla' cells may migrate several millimetres, or even centimetres, away from the major tumour mass. These guerilla cells respond to as yet unknown local environment cues and return to the cell cycle to form recurrent tumours [Pilkington GJ, Brain Pathol.1994; 4: 157-166]. The invasive, disseminating cells at the tumour boundary are extremely unlikely to be dividing and are highly refractory to radiotherapy. Furthermore, ionising radiation is only effective against dividing cells that may form a relatively low proportion of the tumour mass. In addition the blood-brain barrier is often disrupted at the site of the principal tumour mass [Sato S *et al*, Acta Neurochim (Wien) 1998; 140: 1135-1141]. However, delivery of cytotoxic drugs to the invading cells is often inadequate due to the presence of an intact blood-brain barrier in the infiltrated tissue. Consequently, neither mode of therapy is satisfactory and recurrence at the site of the primary lesion is almost inevitable, thus leading to a poor prognosis for the patient.

Invasion is a multi-step process including adhesion of tumour cells to normal brain elements and to extracellular matrix (ECM) components, degradation and remodeling of the ECM by proteases (such as matrix metalloproteinases) and migration [Liotta

LA *et al*, Cancer Res 1986; 46: 1-7]. Matrix metalloproteinases (MMPs) constitute a large family of zinc-dependent endopeptidases that co-operatively degrade all components of the ECM. Consistent with these proteolytic activities, MMPs have been implicated in a variety of physiological and pathological conditions, such as normal embryogenesis, tissue morphogenesis, tumour cell invasion and metastasis [Aznavoorian S *et al*, 1993; 71: 1368-1383; Mignatti P *et al*, 1993; 73: 161-185; Woessner JF, *FASEB* 1991; 5: 2145-2154]. At least 23 MMPs have now been described [Nagase H *et al*, 1999. 274, 21491-21494; Woessner JF, eds WC Parks and RP Mecham, Matrix Metalloproteinases, Academic Press, San Diego. 1998, pp1-14]. Since the initial work by Liotta *et al,* [Nature 1980; 284: 67-68] on MMPs, several members of the MMP family have been implicated in tumour invasion [Cottam DW *et al*, Int J Oncol 1993; 2: 861-872]. Much attention has been drawn to the gelatinases (MMP-2 and -9) in invasion since these enzymes can degrade type IV collagen. There is abundant evidence for an up-regulation of these MMPs in human brain tumours in the literature [Forsyth PA *et al*, J Neurooncol 1998; 36: 21-29; Rao JS *et al*, 1993; 53: 2208-2211; Rooprai HK *et al*, Anticancer Res 1997; 17: 4151-4162; Sawaya RE *et al*, Clin Exp Metastasis 1996; 14: 35-42; Saxena A *et al*, Int J Oncol 1995; 7: 35-42].

The use of anti-invasive agents has been considered as a possible therapeutic approach in addition to conventional surgery and radio-/chemotherapy. Factors implicated in invasion such as extracellular matrix (ECM) components and their cell surface receptors, cell-cell adhesion molecules, proteases and their inhibitors all consist of either secreted or integral membrane glycoproteins. As a consequence, glycoproteins have been highlighted as possible targets for anti-invasion therapy.

In one aspect, the present invention thus seeks to alleviate some of the problems associated with prior art treatments of brain tumours. More particularly, the invention seeks to provide therapeutic agents, preferably anti-invasive agents, for the treatment of brain cancers, which may be used either alone or in combination with conventional surgery and radio-/chemotherapy.

The present invention also relates to the field of neurodegenerative diseases, for example, Parkinson's disease (PD).

Parkinson's disease (PD) is a progressive neurodegenerative disorder affecting motor functions including walking and posture and has been associated with selective loss of pigmented neurons of the substantia nigra area [Pakkenberg B *et al*, J Neurol Neuroserg Psychiat 1991, 54: 30-33]. Although evidence for the role of environmental factors such as exposure to metals, pesticides, or drinking well water in PD is reproducible [Gorell JM *et al*, Neurology 1997, 48: 650-658; Liou HH *et al*, Neurology 1997, 48: 1583-1588.], evidence for genetic linkage with α-synuclein [Golbe LI Mov Disord 1999, 14: 6-9], MAO-B [Mellick GD *et al*, Mov Disord 1999, 14: 219-224] and CYP2D6 to PD is inconclusive [Sabbagh N *et al*, Mov Disord 1999, 14: 230-236]. However, in may neurological studies, neurodegeneration has conclusively been attributed to increased oxidative stress [Simonian NA *et al*, Ann Rev Pharmacol Toxicol 1996, 36: 83-106; 1996; Jenner P (1998), Mov Disord 13: 24-34].

Oxidative stress results when cells are no longer able to control the levels of reactive oxygen radicals. Reactive oxygen species (ROS) are mainly superoxide ($O_2^{-\bullet}$) and hydroxyl radicals ($OH^{\bullet}$) which are generated in all cells during metabolism. Superoxide radicals can be leaked from the mitochondrial electron transport chain, or formed from the activities of enzymes such as xanthine oxidase, cytochrome P450 and phospholipaseA_2. Hydroxyl radicals are formed from H_2O_2 in the presence of reduced metal ions such as Fe^{++}/Cu^{+} via the Fenton reaction or nitric oxide-superoxide reactions.

Increased levels of ROS can damage various cellular components like lipids, proteins and DNA. Selective permeability and potentials of the cell membranes, needed for the survival of cells, are lost when lipids are oxidized, while the oxidation of proteins results in either the activation or inactivation of enzymes by disrupting normal functioning. In turn, this may result in cell death.

Superoxide dismutase and reduced glutathione are part of cellular antioxidant defense system. Superoxide dismutase converts $O_2^{-\bullet}$ to H_2O_2 and reduced glutathione converts H_2O_2 to H_2O. Consequently, oxidative stress may result when there is an increased generation of ROS, or a decreased antioxidant capacity of the cells, or both.

Numerous studies on PD have reported all the hall marks of increased oxidative stress. For example, increased iron deposition was observed in the substantia nigra (involved in generation of free radicals via Fenton reactions) [Dexter DT *et al*, J Neurochem 1989, 52: 1830-1836; Sofic E *et al*, J Neurochem 1991, 56: 978-982], lipid peroxidation [Dexter DT *et al*, J Neurochem1989, 52: 1381-389], protein oxidation [Alam ZI *et al*, J Neurochem 1997, 69: 1196-1203], DNA damage [Sanchez-Ramos JR *et al*, Neurodegeneration 1994, 3: 197-204], decreased reduced glutathione levels [Sian J *et al*, Ann Neurol 1994, 36: 348-355; Sofic E *et al*, Neurosci Lett 1992, 142: 128-130] and increased superoxide dismutase levels [Yoshida E *et al*, J Neurol Sci 1994, 124: 25-31; Yoritaka A *et al*, J Neurol Sci 1997, 148: 181-186] in PD compared to control subjects.

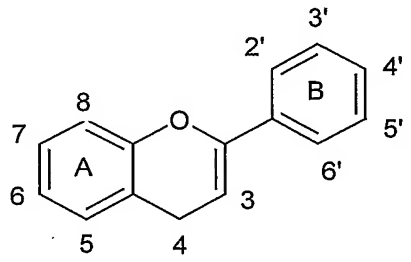
Conversely, the incidence of PD in population groups taking high levels of antioxidants such as vitamin C and E in their diet is low [Tanner CM *et al*, Ann Neurol 1988, 23: 182; De Rijk MC *et al*, Arch Neurol 1997, 54: 762-765] while an increased risk of PD is associated with increased fat intake [Davies KN *et al*, Age aging 1994, 23: 142-145].

Another aspect of the present invention thus seeks to utilise antioxidants as potential therapeutic agents in PD. To date, in spite of compelling evidence for the increased oxidative damage in PD, no therapeutic approaches have been successful in preventing progressive neuronal loss in PD.

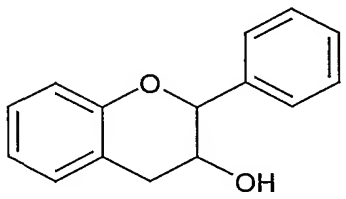
In one aspect, the invention provides a pharmaceutical composition for use (or when in use) in the treatment of brain disorders, said composition comprising one or more flavonoids admixed with a pharmaceutically acceptable diluent, excipient or carrier.

Typically, the brain disorder may be a brain tumour, a neurodegenerative disease, or a neuroinflammatory disease, for example, multiple sclerosis, which has both a neuroinflammatory component and an oxidative damage component.

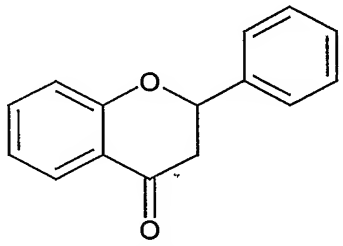
Typically, flavonoids have the basic core structure shown below:



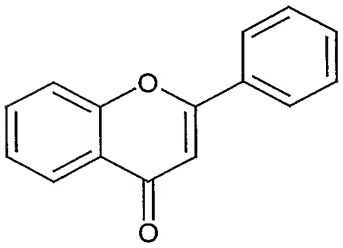
As used herein, the term "flavonoid" also encompasses the following subclasses:



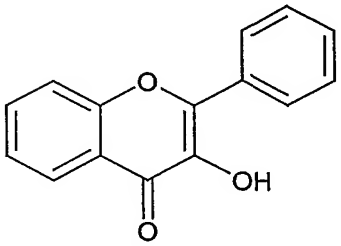
Flavan-3-ol



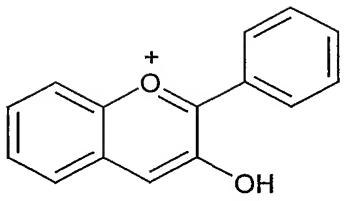
Flavanone



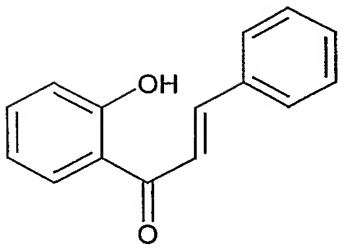
Flavone



Flavon-3-ol



Anthocyanidin



Chalcone

As used herein, the term "flavonoid" is intended to encompass isomeric forms (such as stereoisomers and/or geometric and/or optical isomers, and mixtures thereof), chemical derivatives, mimetics, solvates and salts of flavonoids.

In a preferred embodiment of the invention the flavonoid is derived from one or more of the following: a berry fruit, a citrus fruit, turmeric, green tea, grapes, grapeseed and soya.

In one preferred embodiment, the flavonoid is derived from grapeseed, one such example being proanthocyanidin.

In another preferred embodiment, the flavonoid is derived from soya, one such example being daidzein.

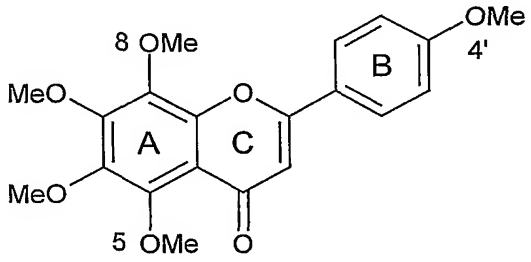
In a particularly preferred embodiment, the flavonoid is derived from a berry fruit.

Even more preferably, the flavonoid is derived from elderberry, chokeberry or bilberry.

In another particularly preferred embodiment, the flavonoid is derived from a citrus fruit.

Even more preferably, the flavonoid is tangeretin or nobiletin.

Tangeretin is a flavonoid present in the peel of citrus fruits [Nelson EK, J Am Chem Soc 1934, 56: 1392-1393] and is a minor constituent of our diet. Tangeretin has the 5,6,7,8,4′ pentamethoxyflavone structure shown below.



Various metabolites of tangeretin are also known and have the chemical structures described below [Nielsen SE *et al*, Food Chem Toxicol 2000, 38: 739-746].

T1	structure not fully elucidated
T2	structure not fully elucidated
T3	4′,6,7-trihydroxy-5,8-dimethoxy-flavone
T4	4′,7-dihydroxy-5,6,8-trimethoxy-flavone
T5	4′,6-dihydroxy-5,7,8-trimethoxy-flavone
T6	3′,4′-dihydroxy-5,6,7,8-tetramethoxy-flavone
T7	4′-hydroxy-5,6,7,8,-tetramethoxy-flavone
T8	structure not fully elucidated
T9	6-hydroxy-4′,5,7,8-tetramethoxy-flavone
T10	5,6-dihydroxy-4′,7,8-trimethoxy-flavone

The present invention is also intended to encompass metabolites of tangeretin (and other flavonoids), such as those listed above.

In addition, the pharmaceutical composition of the invention may further comprise one or more other pharmaceutical or pharmacologically active agents. Other such agents may include, for example, soy isoflavins such as genistein, antioxidant

vitamins A, C, D and E, flavolignans, lectins, selenium, carotenoids such as lycopene, lutein, alpha- and beta-carotene, cryptoxanthin.

Preferably, the flavonoid of the invention is capable of crossing the blood-brain barrier.

In one preferred embodiment of the invention, the brain disorder is a neurodegenerative disorder.

In another preferred embodiment of the invention, the brain disorder is a tumour.

A second aspect of the invention relates to the use of one or more flavonoids in the preparation of a medicament for treating brain disorders, wherein said brain disorders are as described hereinbefore.

As used herein the phrase "preparation of a medicament" includes the use of a compound of the invention directly as the medicament in addition to its use in a screening programme for the identification of further agents or in any stage of the manufacture of such a medicament.

Preferably, the flavonoid is as described hereinbefore and is capable of crossing the blood-brain barrier.

A third aspect of the invention relates to a process for the preparation of a pharmaceutical composition of the invention as described hereinbefore, said process comprising admixing one or more flavonoids with a pharmaceutically acceptable diluent, excipient or carrier.

A fourth aspect of the invention provides a method of treatment for a brain disorder, said method comprising administering to a subject in need of treatment a therapeutically effective amount of one or more flavonoids.

In one preferred embodiment, the invention provides a method of treating a brain disorder, said method comprising administering to a subject in need of treatment a pharmaceutical composition as defined hereinbefore.

It is to be appreciated that all references herein to treatment include one or more of curative, palliative and prophylactic treatment. Preferably, the term treatment includes at least curative treatment and/or palliative treatment.

A fifth aspect of the invention provides a pharmaceutical pack for use in the treatment of brain disorders; the pack comprising one or more compartments; wherein at least one of said compartments houses one or more flavonoids. In the pack of the present invention, the flavonoid may be admixed with a pharmaceutically acceptable carrier, diluent or excipient. In addition, or in the alternative, the pack of the present invention may comprise a further compartment which houses a pharmaceutically acceptable carrier, diluent or excipient.

In a preferred embodiment, at least one of said compartments houses one or more flavonoids, and at least one of the other compartments of the pack comprises one or more other pharmaceutically active agents.

Where the pack of the present invention comprises more than one flavonoid, the flavonoids may be in different forms. Likewise, where the pack of the invention comprises one or more flavonoids together with one or more other pharmaceutically active agents, the one or more flavonoids and the other pharmaceutically active agents may be in different forms. By way of example, one may be a solution or tablet and the other may be a cream. In one preferred embodiment of the present invention, one component of the pack is to be applied topically and the other component of the pack is to be applied systemically. It is to be understood that the pack could contain extra compartments.

A sixth aspect of the invention provides an assay method for identifying an agent that can directly or indirectly downregulate the expression of MMP-2 and/or MMP-9 in order to treat brain disorders, preferably brain tumours, the assay method comprising: contacting an agent with MMP-2 and/or MMP-9; and measuring the activity of MMP-2 and/or MMP-9; wherein a downregulation of expression of MMP-2 and/or MMP-9 in the presence of the agent is indicative that the agent may be useful in the treatment of brain disorders, preferably brain tumours.

The invention further relates to a process comprising the steps of:

(a) performing the above-mentioned assay;

(b) identifying one or more agents that can directly or indirectly downregulate the expression of MMP-2 and/or MMP-9; and

(c) preparing a quantity of those one or more identified agents.

In addition, another aspect of the invention relates to a method of treating brain disorders, by downregulating expression of MMP-2 and/or MMP-9 *in vivo* with an agent;
wherein the agent is capable of directly or indirectly downregulating expression of MMP-2 and/or MMP-9 in an *in vitro* assay method;
wherein the *in vitro* assay method is as described hereinbefore.

Yet another aspect of the invention relates to the use of an agent in the preparation of a pharmaceutical composition for the treatment of brain disorders, preferably brain tumours, wherein the agent is capable of directly or indirectly downregulating expression of MMP-2 and/or MMP-9 when assayed *in vitro* by the assay method described above.

The invention also relates to an agent identified by the assay method described above.

Preferably, the agent identified by the assay method described above is for use in medicine.

Even more preferably, the agent identified by the assay method described above is for use in treating brain cancer.

Another aspect of the invention relates to the use of one or more flavonoids in an assay for identifying candidate compounds that are capable of influencing the expression of MMP-2 and/or MMP-9.

FLAVONOIDS

Flavonoids are a group of compounds which are ubiquitous in nature and are an integral part of the human diet. In particular, flavonoids are present in vegetables, fruits, nuts, seeds, grain, wine and tea [Hertog MGL *et al*, Nutr Cancer 1993; 20: 21-29; Kuhnau J, World Rev Nutr Diet 1976; 24: 117-119] and may be broadly classified as phenolic or non-phenolic. It is estimated that the daily intake of flavonoids in the Western diet ranges from 20 mg/day to 1 g/day. Over 5000 different naturally-occurring flavonoids have been described [Harborne JB (ed.) The flavonoids, 1994; Advances in Research since 1986, Chapman and Hall, London], all of which are derivatives of the parent compound flavone (2-phenyl-benzopyrene or 2-phenylchromone) which comprises a tricyclic C_6-C_3-C_6 nucleus. Varying the substituents of the parent flavone gives rise to nine classes of flavonoid compounds.

Flavonoids exhibit a broad spectrum of pharmacological effects ranging from vasodilatory, anti-inflammatory, antibacterial, anti-allergic, antiviral to anti-carcinogenic and immunostimulant activities [Hollman PCH *et al*, Biomed Pharmacother 1997, 51: 305-310; Middleton E *et al*, Biochem 1992, 43: 1167-1179]. Aside from having this broad spectrum of pharmacological activity, many flavonoids are better antioxidants than vitamin C or E on a mole to mole basis [Rice-Evans CA *et al*, Free Rad Res 1995, 22: 375-3831995; Castelluccio C *et al*, FEBB Lett 1995, 368: 188-192]. Further information on the antioxidant properties of flavonoids is currently

emerging. For example, depending on the position of hydroxyl groups [van Acker SABE *et al*, Free Rad Biol Med 1996, 20: 331-342; Arora A *et al*, Free Rad Biol Med 1998, 42: 1355-1363] some flavonoids have more free radical scavenging activity [Robak J *et al*, Biochem Pharmacol 1988, 37: 837-841; Saija A *et al*, Free Rad Biol Med 1995, 19: 481-486], or metal chelating properties [Morel I *et al*, Biochem Pharmacol 1993, 45: 13-19] or have the ability to increase the expression of antioxidant proteins [Cai Q *et al*, Nutr Cancer 1996, 25: 1-7; Kuo SM *et al*, Biol Trace Elem Re 1998, 62: 135-153; Sudheesh S *et al*, Phytother Res 1999, 13: 393-396].

STEREO AND GEOMETRIC ISOMERS

Some of the flavonoids may exist as stereoisomers and/or geometric isomers – e.g. they may possess one or more asymmetric and/or geometric centres and so may exist in two or more stereoisomeric and/or geometric forms. The present invention contemplates the use of all the individual stereoisomers and geometric isomers of those flavonoids, and mixtures thereof. The terms used in the claims encompass these forms, provided said forms retain the appropriate functional activity (though not necessarily to the same degree).

SOLVATES

The present invention also includes the use of solvate forms of the flavonoid of the present invention. The terms used in the claims encompass these forms.

PRO-DRUG

As indicated, the present invention also includes the use of pro-drug forms of the flavonoids of the present invention. The terms used in the claims encompass these forms. Examples of prodrugs include entities that have certain protected group(s) and which may not possess pharmacological activity as such, but may, in certain instances, be administered (such as orally or parenterally) and thereafter metabolised

in the body to form the flavonoids of the present invention which are pharmacologically active.

It will be further appreciated that certain moieties known as "pro-moieties", for example as described in "Design of Prodrugs" by H. Bundgaard, Elsevier, 1985 (the disclosured of which is hereby incorporated by reference), may be placed on appropriate functionalities of the flavonoids. Such prodrugs are also included within the scope of the invention.

MIMETIC

In one embodiment of the present invention, the flavonoid may be a flavonoid mimetic. As used herein, the term "mimetic" relates to any chemical which includes, but is not limited to, a peptide, polypeptide, antibody or other organic chemical which has the same qualitative activity or effect as a reference agent .

CHEMICAL DERIVATIVE

In one embodiment of the present invention, the flavonoid may be a derivative of a flavonoid. The term "derivative" as used herein includes chemical modification of a flavonoid. Illustrative of such chemical modifications would be replacement of hydrogen by a halo group, an alkyl group, an acyl group or an amino group.

CHEMICAL MODIFICATION

In one embodiment of the present invention, the flavonoid may be a chemically modified flavonoid.

The chemical modification of a flavonoid of the present invention may either enhance or reduce hydrogen bonding interaction, charge interaction, hydrophobic interaction, Van Der Waals interaction or dipole interaction between the agent and the target.

PHARMACEUTICAL SALTS

The flavonoids of the present invention may be administered as pharmaceutically acceptable salts. Typically, a pharmaceutically acceptable salt may be readily prepared by using a desired acid or base, as appropriate. The salt may precipitate from solution and be collected by filtration or may be recovered by evaporation of the solvent.

Pharmaceutically-acceptable salts are well known to those skilled in the art, and for example include those mentioned by Berge *et al*, in J.Pharm.Sci., 66, 1-19 (1977). Suitable acid addition salts are formed from acids which form non-toxic salts and include the hydrochloride, hydrobromide, hydroiodide, nitrate, sulphate, bisulphate, phosphate, hydrogenphosphate, acetate, trifluoroacetate, gluconate, lactate, salicylate, citrate, tartrate, ascorbate, succinate, maleate, fumarate, gluconate, formate, benzoate, methanesulphonate, ethanesulphonate, benzenesulphonate and p-toluenesulphonate salts.

When one or more acidic moieties are present, suitable pharmaceutically acceptable base addition salts can be formed from bases which form non-toxic salts and include the aluminium, calcium, lithium, magnesium, potassium, sodium, zinc, and pharmaceutically-active amines such as diethanolamine, salts.

The flavonoids of the present invention may exist in polymorphic form.

In addition, the flavonoids of the present invention may contain one or more asymmetric carbon atoms and therefore exists in two or more stereoisomeric forms. Where a flavonoid contains an alkenyl or alkenylene group, cis (E) and trans (Z) isomerism may also occur. The present invention includes the individual stereoisomers of the flavonoid and, where appropriate, the individual tautomeric forms thereof, together with mixtures thereof.

Separation of diastereoisomers or cis and trans isomers may be achieved by conventional techniques, e.g. by fractional crystallisation, chromatography or H.P.L.C. of a stereoisomeric mixture of the agent or a suitable salt or derivative thereof. An individual enantiomer of the flavonoid may also be prepared from a corresponding optically pure intermediate or by resolution, such as by H.P.L.C. of the corresponding racemate using a suitable chiral support or by fractional crystallisation of the diastereoisomeric salts formed by reaction of the corresponding racemate with a suitable optically active acid or base, as appropriate.

The present invention also includes all suitable isotopic variations of the flavonoid or a pharmaceutically acceptable salt thereof. An isotopic variation of an flavonoid of the present invention or a pharmaceutically acceptable salt thereof is defined as one in which at least one atom is replaced by an atom having the same atomic number but an atomic mass different from the atomic mass usually found in nature. Examples of isotopes that can be incorporated into the flavonoid and pharmaceutically acceptable salts thereof include isotopes of hydrogen, carbon, nitrogen, oxygen, phosphorus, sulphur, fluorine and chlorine such as ^{2}H, ^{3}H, ^{13}C, ^{14}C, ^{15}N, ^{17}O, ^{18}O, ^{31}P, ^{32}P, ^{35}S, ^{18}F and ^{36}Cl, respectively. Certain isotopic variations of the flavonoid and pharmaceutically acceptable salts thereof, for example, those in which a radioactive isotope such as ^{3}H or ^{14}C is incorporated, are useful in drug and/or substrate tissue distribution studies. Tritiated, i.e., ^{3}H, and carbon-14, i.e., ^{14}C, isotopes are particularly preferred for their ease of preparation and detectability. Further, substitution with isotopes such as deuterium, i.e., ^{2}H, may afford certain therapeutic advantages resulting from greater metabolic stability, for example, increased *in vivo* half-life or reduced dosage requirements and hence may be preferred in some circumstances. Isotopic variations of the flavonoid of the present invention and pharmaceutically acceptable salts thereof of this invention can generally be prepared by conventional procedures using appropriate isotopic variations of suitable reagents.

The present invention also includes (wherever appropriate) the use of zwitterionic forms of the flavonoids of the present invention.

The terms used in the claims encompass one or more of the forms just mentioned.

FORMULATION

The component(s) of the present invention may be formulated into a pharmaceutical composition, such as by mixing with one or more of a suitable carrier, diluent or excipient, by using techniques that are known in the art.

PHARMACEUTICAL COMPOSITIONS

The present invention provides a pharmaceutical composition comprising a therapeutically effective amount of one or more flavonoids and a pharmaceutically acceptable carrier, diluent or excipient (including combinations thereof).

The pharmaceutical compositions may be for human or animal usage in human and veterinary medicine and will typically comprise any one or more of a pharmaceutically acceptable diluent, carrier, or excipient. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985). The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s).

Examples of suitable carriers include lactose, starch, glucose, methyl cellulose, magnesium stearate, mannitol, sorbitol and the like. Examples of suitable diluents include ethanol, glycerol and water.

Examples of suitable binders include starch, gelatin, natural sugars such as glucose, anhydrous lactose, free-flow lactose, beta-lactose, corn sweeteners, natural and synthetic gums, such as acacia, tragacanth or sodium alginate, carboxymethyl cellulose and polyethylene glycol.

Examples of suitable lubricants include sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like.

Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. Examples of preservatives include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. Antioxidants and suspending agents may be also used.

There may be different composition/formulation requirements dependent on the different delivery systems. By way of example, the pharmaceutical composition of the present invention may be formulated to be administered using a mini-pump or by a mucosal route, for example, as a nasal spray or aerosol for inhalation or ingestable solution, or parenterally in which the composition is formulated by an injectable form, for delivery, by, for example, an intravenous, intramuscular or subcutaneous route. Alternatively, the formulation may be designed to be administered by a number of routes.

Where the composition is to be administered mucosally through the gastrointestinal mucosa, it should be able to remain stable during transit though the gastrointestinal tract; for example, it should be resistant to proteolytic degradation, stable at acid pH and resistant to the detergent effects of bile.

Where appropriate, the pharmaceutical compositions can be administered by inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile

aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

For some embodiments, one or more flavonoids may also be used in combination with a cyclodextrin. Cyclodextrins are known to form inclusion and non-inclusion complexes with drug molecules. Formation of a drug-cyclodextrin complex may modify the solubility, dissolution rate, bioavailability and/or stability property of a drug molecule. Drug-cyclodextrin complexes are generally useful for most dosage forms and administration routes. As an alternative to direct complexation with the drug the cyclodextrin may be used as an auxiliary additive, e.g. as a carrier, diluent or solubiliser. Alpha-, beta- and gamma-cyclodextrins are most commonly used and suitable examples are described in WO-A-91/11172, WO-A-94/02518 and WO-A-98/55148.

CHEMICAL SYNTHESIS METHODS

Typically the flavonoids of the present invention are available commercially, and are prepared by extraction processes from the appropriate flavonoid-containing fruit or vegetable. Details of such extraction methods may be found in W W Widmer and A M Montanari [Proc. Fla. State Hort. Soc. 1994, 107: 284-288].

Alternatively, the flavonoid of the invention may be prepared by chemical synthesis techniques.

It will be apparent to those skilled in the art that sensitive functional groups may need to be protected and deprotected during synthesis of a compound of the invention. This may be achieved by conventional techniques, for example as described in "Protective Groups in Organic Synthesis" by T W Greene and P G M Wuts, John Wiley and Sons Inc. (1991), and by P.J.Kocienski, in "Protecting Groups", Georg Thieme Verlag (1994).

It is possible during some of the reactions that any stereocentres present could, under certain conditions, be racemised, for example if a base is used in a reaction with a substrate having an having an optical centre comprising a base-sensitive group. It should be possible to circumvent potential problems such as this by choice of reaction sequence, conditions, reagents, protection/deprotection regimes, etc. as is well-known in the art.

The flavonoids and salts of the invention may be separated and purified by conventional methods.

Separation of diastereomers may be achieved by conventional techniques, e.g. by fractional crystallisation, chromatography or H.P.L.C. of a stereoisomeric mixture of a flavonoid or a suitable salt or derivative thereof. An individual enantiomer of a flavonoid may also be prepared from a corresponding optically pure intermediate or by resolution, such as by H.P.L.C. of the corresponding racemate using a suitable chiral support or by fractional crystallisation of the diastereomeric salts formed by reaction of the corresponding racemate with a suitably optically active acid or base. Alternatively, the flavonoid may be used as the racemate, or as a mixture of enantiomers.

THERAPY

The agents identified by any such assay method may be used as therapeutic agents – i.e. in therapy applications.

As with the term "treatment", the term "therapy" includes curative effects, alleviation effects, and prophylactic effects.

The therapy may be on humans or animals.

ADMINISTRATION

The components of the present invention may be administered alone but will generally be administered as a pharmaceutical composition – e.g. when the

components are is in admixture with a suitable pharmaceutical excipient, diluent or carrier selected with regard to the intended route of administration and standard pharmaceutical practice.

For example, the composition can be administered (e.g. orally or topically) in the form of tablets, capsules, ovules, elixirs, solutions or suspensions, which may contain flavouring or colouring agents, for immediate-, delayed-, modified-, sustained-, pulsed- or controlled-release applications.

If the pharmaceutical composition is a tablet, then the tablet may contain excipients such as microcrystalline cellulose, lactose, sodium citrate, calcium carbonate, dibasic calcium phosphate and glycine, disintegrants such as starch (preferably corn, potato or tapioca starch), sodium starch glycollate, croscarmellose sodium and certain complex silicates, and granulation binders such as polyvinylpyrrolidone, hydroxypropylmethylcellulose (HPMC), hydroxypropylcellulose (HPC), sucrose, gelatin and acacia. Additionally, lubricating agents such as magnesium stearate, stearic acid, glyceryl behenate and talc may be included.

Solid compositions of a similar type may also be employed as fillers in gelatin capsules. Preferred excipients in this regard include lactose, starch, cellulose, milk sugar or high molecular weight polyethylene glycols. For aqueous suspensions and/or elixirs, the flavonoid may be combined with various sweetening or flavouring agents, colouring matter or dyes, with emulsifying and/or suspending agents and with diluents such as water, ethanol, propylene glycol and glycerin, and combinations thereof.

The routes for administration (delivery) include, but are not limited to, one or more of: oral (e.g. as a tablet, capsule, or as an ingestable solution), topical, mucosal (e.g. as a nasal spray or aerosol for inhalation), nasal, parenteral (e.g. by an injectable form), gastrointestinal, intraspinal, intraperitoneal, intramuscular, intravenous, intrauterine, intraocular, intradermal, intracranial, intratracheal, intravaginal, intracere-

broventricular, intracerebral, subcutaneous, ophthalmic (including intravitreal or intracameral), transdermal, rectal, buccal, vaginal, epidural, sublingual.

Where the composition comprises more than one flavonoid, it is to be understood that not all of the components of the pharmaceutical need be administered by the same route. Likewise, if the composition comprises more than one active component, then those components may be administered by different routes.

If a component of the present invention is administered parenterally, then examples of such administration include one or more of: intravenously, intra-arterially, intraperitoneally, intrathecally, intraventricularly, intraurethrally, intrasternally, intracranially, intramuscularly or subcutaneously administering the component; and/or by using infusion techniques.

For parenteral administration, the component is best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well-known to those skilled in the art.

As indicated, the component(s) of the present invention can be administered intranasally or by inhalation and is conveniently delivered in the form of a dry powder inhaler or an aerosol spray presentation from a pressurised container, pump, spray or nebuliser with the use of a suitable propellant, e.g. dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, a hydrofluoroalkane such as 1,1,1,2-tetrafluoroethane (HFA 134A™) or 1,1,1,2,3,3,3-heptafluoropropane (HFA 227EA™), carbon dioxide or other suitable gas. In the case of a pressurised aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. The pressurised container, pump, spray or nebuliser may contain a solution or suspension of the active compound, e.g. using a mixture of ethanol and the propellant

as the solvent, which may additionally contain a lubricant, e.g. sorbitan trioleate. Capsules and cartridges (made, for example, from gelatin) for use in an inhaler or insufflator may be formulated to contain a powder mix of the agent and a suitable powder base such as lactose or starch.

Alternatively, the component(s) of the present invention can be administered in the form of a suppository or pessary, or it may be applied topically in the form of a gel, hydrogel, lotion, solution, cream, ointment or dusting powder. The component(s) of the present invention may also be dermally or transdermally administered, for example, by the use of a skin patch. They may also be administered by the pulmonary or rectal routes. They may also be administered by the ocular route. For ophthalmic use, the compounds can be formulated as micronised suspensions in isotonic, pH adjusted, sterile saline, or, preferably, as solutions in isotonic, pH adjusted, sterile saline, optionally in combination with a preservative such as a benzylalkonium chloride. Alternatively, they may be formulated in an ointment such as petrolatum.

For application topically to the skin, the component(s) of the present invention can be formulated as a suitable ointment containing the active compound suspended or dissolved in, for example, a mixture with one or more of the following: mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene polyoxypropylene compound, emulsifying wax and water. Alternatively, it can be formulated as a suitable lotion or cream, suspended or dissolved in, for example, a mixture of one or more of the following: mineral oil, sorbitan monostearate, a polyethylene glycol, liquid paraffin, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water.

In a preferred embodiment of the invention, the pharmaceutical composition is administered orally.

DOSE LEVELS

Typically, a physician will determine the actual dosage which will be most suitable for an individual subject. The specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors including the activity of the specific compound employed, the metabolic stability and length of action of that compound, the age, body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular condition, and the individual undergoing therapy.

Depending upon the need, the agent may be administered at a dose of from 0.01 to 30 mg/kg body weight, such as from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

MMP

MMPs constitute a family of structurally similar zinc-containing metalloproteases, which are involved in the remodelling, repair and degradation of extracellular matrix proteins, both as part of normal physiological processes and in pathological conditions. At least 18 members of the human family have been sequenced.

Since they have high destructive potential, the MMPs are usually under close regulation, and failure to maintain MMP regulation has been implicated as a component of a number of conditions. Examples of conditions where MMPs are thought to be important are those involving bone restructuring, embryo implantation in the uterus, infiltration of immune cells into inflammatory sites, ovulation, spermatogenesis, tissue remodelling during wound repair and organ differentiation such as such as in venous and diabetic ulcers, pressure sores, colon ulcers for example ulcerative colitis and Crohn's disease, duodenal ulcers, fibrosis, local invasion of tumours into adjacent areas, metastatic spread of tumour cells from primary to secondary sites, and tissue destruction in arthritis, skin disorders such as dystrophic epidermolysis bulosa, dermatitis herpetiformis, or conditions caused by or

complicated by embolic phenomena, such as chronic or acute cardiac or cerebral infarctions.

Substrates for the MMPs are diverse and sometimes include other members of the gene family. For example, MMP-14 is known to digest and activate proMMP-2 and both MMP-3 and MMP-9 can digest and activate proMMP-1. Some MMP substrates are also matrix components - such as collagen which is digested, for example by MMP-1 (also known as collagenase-1), denatured collagen or gelatin which is digested for example, by MMP-2 (also known as gelatinase-A), fibronectin which is digested for example by MMP-3 (allso known as stromelysin-1) and glycosaminoglycans which is digested for example by MMP-3.

For recent reviews of MMPs, see Zask *et al*, Current Pharmaceutical Design, **1996**, *2*, 624-661; Beckett, Exp. Opin. Ther. Patents, **1996**, *6*, 1305-1315; and Beckett *et al*, Drug Discovery Today, vol 1(no.1), 1996, 16-26.

The teachings of the present invention are particularly concerned with MMP-2 and MMP-9. Alternative names for these MMPs and substrates acted on by these are shown in the table below (Zask *et al, supra*).

Enzyme	**Other names**	**Preferred substrates**
MMP-2	Gelatinase A, 72kDa gelatinase	Gelatins, collagens IV, V, VII, X, elastin, fibronectin; activates pro-MMP-13
MMP-9	Gelatinase B, 92 kDa gelatinase	Gelatins, collagens IV, V, elastin

MMP2

Background teachings on matrix metalloproteinase 2 (MMP2) have been presented by Victor A. McKusick *et al* on http://www.ncbi.nlm.nih.gov/Omim. For ease of reference, the following information has been extracted from that source.

"Type IV collagenase is a metalloproteinase that specifically cleaves type IV collagen, the major structural component of basement membranes. The metastatic potential of tumor cells has been found to correlate with the activity of this enzyme.

Huhtala *et al.* (1990) determined that the CLG4A gene is 17 kb long with 13 exons varying in size from 110 to 901 bp and 12 introns ranging from 175 to 4,350 bp. Alignment of introns showed that introns 1 to 4 and 8 to 12 of the type IV collagenase gene coincide with intron locations in the interstitial collagenase and stromelysin genes, indicating a close structural relationship of these metalloproteinase genes. Devarajan *et al.* (1992) reported on the structure and expression of 78-kD gelatinase, which they referred to as neutrophil gelatinase.

Type IV collagenase, 72-kD, is officially designated matrix metalloproteinase-2 (MMP2). It is also known as gelatinase, 72-kD (Nagase *et al.*, 1992).

Irwin *et al.* (1996) presented evidence that MMP2 is a likely effector of endometrial menstrual breakdown. They cultured human endometrial stromal cells in the presence of progesterone and found an augmentation of proteinase production after withdrawal of proteinase: the same results were achieved by the addition of the P receptor antagonist RU486. Characterization of the enzyme by Western blotting revealed it to be MMP2. Northern blot analysis showed differential expression of MMP2 mRNA in late secretory phase endometrium.

Angiogenesis depends on both cell adhesion and proteolytic mechanisms. Matrix metalloproteinase-2 and integrin α-V/β-3 are functionally associated on the surface of angiogenic blood vessels. Brooks *et al.* (1998) found that a fragment of MMP2, which comprises the C-terminal hemopexin-like domain (amino acids 445-635) and is termed PEX, prevents this enzyme from binding to α-V/β-3 and blocks cell surface collagenolytic activity in melanoma and endothelial cells. PEX blocks MMP2 activity on the chick chorioallantoic membrane where it disrupts angiogenesis and tumor growth. Brooks *et al.* (1998) also found that a naturally occurring form of PEX can be detected *in vivo* in conjunction with α-V/β-3 expression in tumors and during developmental retinal neovascularization. Levels of PEX in these vascularized tissues suggest that it interacts with endothelial cell α-V/β-3 where it serves as a natural inhibitor of MMP2 activity, thereby regulating the invasive behavior of new blood vessels. The authors concluded that recombinant PEX may provide a potentially novel therapeutic approach for diseases associated with neovascularization.

By hybridization to a panel of DNAs from human-mouse cell hybrids and by in situ hybridization using a gene probe, Fan *et al.* (1989) assigned the CLG4 gene to 16q21; see Huhtala *et al.* (1990). By hybridization to somatic cell hybrid DNAs, Collier *et al.* (1991) assigned both CLG4A and CLG4B to chromosome 16. Chen *et al.* (1991) mapped 12 genes on the long arm of chromosome 16 by the use of 14 mouse/human hybrid cell lines and the fragile site FRA16B. The breakpoints in the hybrids, in conjunction with the fragile site, divided the long arm into 14 regions. They concluded that CLG4 is in band 16q13.

Morgunova *et al.* (1999) reported the crystal structure of the full-length proform of human MMP2. The crystal structure revealed how the propeptide shields the catalytic cleft and that the cysteine switch may operate through cleavage of loops essential for propeptide stability.

Becker-Follmann *et al.* (1997) created a high-resolution map of the linkage group on mouse chromosome 8 that is conserved on human 16q. The map extended from the homolog of the MMP2 locus on 16q13 (the most centromeric locus) to CTRB on 16q23.2-q23.3."

MMP9

Background teachings on matrix metalloproteinase 9 (MMP9) have been presented by Victor A. McKusick *et al* on http://www.ncbi.nlm.nih.gov/Omim. For ease of reference, the following information has been extracted from that source.

"The 72- and 92-kD type IV collagenases are members of a group of secreted zinc metalloproteases which, in mammals, degrade the collagens of the extracellular matrix. Other members of this group include interstitial collagenase and stromelysin. The 72-kD type IV collagenase is secreted from normal skin fibroblasts, whereas the 92-kD collagenase (CLG4B) is produced by normal alveolar macrophages and granulocytes. Both CLG and STMY have 10 exons of virtually identical length, are located on 11q, and are regulated in a coordinate fashion. By hybridization to somatic cell hybrid DNAs, Collier *et al.* (1991) demonstrated that both CLG4A and CLG4B are situated on chromosome 16. However, St Jean *et al.* (1995) assigned CLG4B to chromosome 20. They did linkage mapping of the CLG4B locus in 10 CEPH reference pedigrees using a polymorphic dinucleotide repeat in the 5-prime flanking region of the gene. St Jean *et al.* (1995) observed lod scores of between 10.45 and 20.29 with markers spanning chromosome region 20q11.2-q13.1. Further support for assignment of CLG4B to chromosome 20 was provided by analysis of human/rodent somatic cell hybrids. Both CLG4A and CLG4B have 13 exons and similar intron locations (Huhtala *et al.*, 1991). Due to these similarities, the CLG4B cDNA clone used in the mapping to chromosome 16 may have hybridized to CLG4A rather than to CLG4B on chromosome 20.

The 13 exons of both CLG4A and CLG4B are 3 more than have been found in other members of this gene family. The extra exons encode the amino acids of the fibronectin-like domain which has been found only in the 72- and 92-kD type IV collagenases. The 92-kD type IV collagenase is also known as 92-kD gelatinase, type V collagenase, or matrix metalloproteinase 9 (MMP9); see the glossary of matrix metalloproteinases provided by Nagase *et al.* (1992).

Linn *et al.* (1996) reassigned MMP9 (referred to as CLG4B by them) to chromosome 20 based on 3 different lines of evidence: screening of a somatic cell hybrid mapping panel,

fluorescence in situ hybridization, and linkage analysis using a newly identified polymorphism. They also mapped mouse Clg4b to mouse chromosome 2, which has no known homology to human chromosome 16 but large regions of homology with human chromosome 20.

By targeted disruption in embryonic stem cells, Vu *et al.* (1998) created homozygous mice with a null mutation in the MMP9/gelatinase B gene. These mice exhibited an abnormal pattern of skeletal growth plate vascularization and ossification. Although hypertrophic chondrocytes developed normally, apoptosis, vascularization, and ossification were delayed, resulting in progressive lengthening of the growth plate to about 8 times normal. After 3 weeks postnatal, aberrant apoptosis, vascularization, and ossification compensated to remodel the enlarged growth plate and ultimately produced an axial skeleton of normal appearance. Transplantation of wildtype bone marrow cells rescued vascularization and ossification in MMP9-null growth plates, indicating that these processes are mediated by MMP9-expressing cells of bone marrow origin, designated chondroclasts. Growth plates from MMP9-null mice in culture showed a delayed release of an angiogenic activator, establishing a role for this proteinase in controlling angiogenesis."

GENERAL ASSAY TECHNIQUES

In one aspect, the identified flavonoid may act as a model (for example, a template) for the development of other compounds. The flavonoids employed in such a test may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The abolition of activity or the formation of binding complexes between the flavonoid and the agent being tested may be measured.

The assay of the present invention may be a screen, whereby a number of agents are tested. In one aspect, the assay method of the present invention is a high through put screen.

Techniques for drug screening may be based on the method described in Geysen, European Patent Application 84/03564, published on September 13, 1984. In summary, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with a suitable flavonoid or fragment thereof and washed. Bound entities are then detected - such as by appropriately adapting methods well

known in the art. A purified flavonoid can also be coated directly onto plates for use in a drug screening techniques. Alternatively, non-neutralising antibodies can be used to capture the peptide and immobilise it on a solid support.

This invention also contemplates the use of competitive drug screening assays in which neutralising antibodies capable of binding a flavonoid specifically compete with a test compound for binding to a flavonoid.

Another technique for screening provides for high throughput screening (HTS) of agents having suitable binding affinity to the substances and is based upon the method described in detail in WO 84/03564.

It is expected that the assay methods of the present invention will be suitable for both small and large-scale screening of test compounds as well as in quantitative assays.

REPORTERS

A wide variety of reporters may be used in the assay methods (as well as screens) of the present invention with preferred reporters providing conveniently detectable signals (eg. by spectroscopy). By way of example, a number of companies such as Pharmacia Biotech (Piscataway, NJ), Promega (Madison, WI), and US Biochemical Corp (Cleveland, OH) supply commercial kits and protocols for assay procedures. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US-A-3817837; US-A-3850752; US-A-3939350; US-A-3996345; US-A-4277437; US-A-4275149 and US-A-4366241.

TREATMENT OF PARKINSON'S DISEASE

As mentioned hereinbefore, in a particularly preferred embodiment, the neurodegenerative disorder is Parkinson's disease. In this regard, the invention seeks to further elucidate (i) whether flavonoids reach the brain by crossing blood brain

barrier (BBB) and, if they do cross the BBB, (ii) whether neuronal loss is prevented as a result of their antioxidant properties.

Experiments were therefore undertaken by the applicant to investigate whether tangeretin can cross the BBB and protect against neuronal loss in a rat model of PD. Further details of these studies are outlined below and in the accompanying Examples. By way of summary, the results provide evidence for the first time of (a) the capacity of tangeretin to cross BBB and (b) the ability of tangeretin to prevent TH+ neuronal loss after 6-OHDA, a model of Parkinson's disease. Indeed, tangeretin levels in the brain were found to be up to 6-fold higher than any other peripheral organ tested, demonstrating that tangeretin is in effect concentrated in the brain.

Tangeretin, when administered orally, was shown to be metabolized into demethylated or hydroxylated metabolites and is excreted through urine and feces. Of the various metabolites, at least 10 have been identified and characterized to date.

Interestingly all the metabolites of tangeretin have similar UV-spectral patterns, implying that studies cannot distinguish whether the levels reported are that of the metabolites or the parent compound or mixtures thereof. As metabolite standards are not yet commercially available, the quantification of individual metabolites by UV-spectroscopy proved difficult [Nielsen *et al*, *ibid*]. In spite of this lack of distinction, the unequal distribution of tangeretin observed in the brain suggests a regional selectivity, with especially high concentrations in the striatal and hypothalamic regions.

The 6-OHDA lesion rat model fulfils the construct validity of Parkinson's disease in that TH+ neuronal loss is progressive as in PD. The model is also characterised by a loss of dopamine in the striatum. Tangeretin pretreatment seems to prevent such neuronal loss. 6-OHDA is known to generate hydroxyl radicals that play a role in the TH+ neuronal loss. Whether tangeretin protects neuronal loss after 6-OHDA lesions by scavenging hydroxyl radicals generated cannot be substantiated from this study.

However, without wishing to be bound by theory, structure activity studies with other flavonoids suggest that the presence of a catechol (two hydroxyl groups on the adjacent carbon atoms) structure on the A-ring may lead to free radical scavenging activity [Arora *et al*, *ibid*].

6-OHDA results in the loss of dopaminergic neurons in the nigro-striatal tract as in PD. The model may be assessed by immunocytochemistry and by counting the number of TH+ neurons in the substantia nigra. In addition to the concentrations of dopamine, the neurotransmittor in the neuronal terminal sites in the striatum may be assessed. Tangeretin not only preserves the dopaminergic cells after 6-OHDA but also preserves dopamine levels in the striatum, again, supporting preservation of the dopaminergic neurons, i.e., indicative of a neuroprotective effect.

TREATMENT OF BRAIN CANCERS

As mentioned above, in another preferred embodiment of the invention, the brain disorder is a tumour. Further studies by the applicant have investigated the effects of the flavonoids tangeretin and nobiletin on the biological behaviour of brain tumour cells *in vitro*.

Tangeretin and nobiletin are non-phenolic citrus flavonoids found in tangerines, lemons and lime. These flavonoids have been investigated by various workers for their anti-proliferative [Guthrie N *et al*, In: Flavonoids in the living system, Eds Manthey and Buslig, Plenum Press New York, 1998: 227-236; Kandaswami C *et al*, Cancer Lett 1991; 56; 147-152; Kawaii S *et al*, Biosci Biotechnol Biochem 1999; 63: 896-899; Mookerjee BK *et al*, J Immunopharmacol 1986; 8: 371-392; So FV *et al*, Nutr Cancer 1996; 26: 167-181] and anti-invasive properties in other cancers [Bracke ME *et al*, Clin Expt Metastasis 1989; 7: 283-3005; Bracke M *et al*, Clin Expt Metastasis 1991; 9: 13-25; Mareel MM *et al*, Critical Reviews Oncol/Haematol 1989; 9: 263-303] but to date there are no reports of their effects on brain tumours.

Although intrinsic tumours of the brain seldom metastasize to distant sites, their diffuse, infiltrative-invasive growth within the brain generally precludes successful surgical and adjuvant therapy. Attention has therefore focused on novel therapeutic approaches to combat brain tumours which includes the use of anti-invasive and anti-proliferative agents. The effect of four anti-invasive agents, swainsonine (a locoweed alkaloid), captopril (an antihypertensive drug), tangeretin and nobiletin (both citrus flavonoids), have been investigated on various parameters of brain tumour invasion such as matrix metalloproteinase (MMP) secretion, *in vitro* migration, invasion and adhesion.

Captopril is an angiotensin converting enzyme (ACE) inhibitor that has been widely used as an antihypertensive drug. ACE belongs to a class of metalloproteinases similar to MMPs. Reports of the effect of captopril on brain tumour invasion are very limited: Nakagawa *et al,* [Anticancer Res 1995; 15: 1985-1990] have shown that it inhibits the invasiveness of T98G glioma cells as a result of MMP inhibitory activity, by chelating zinc ions at the enzyme's active site.

Swainsonine is an indolizidine derived from the plant *Swainsona canescens* [Dorling PR *et al*, Biochem J 1980; 191: 649-651] which is an inhibitor of lysosomal alpha-mannosidase. The potential use of swainsonine as an anticancer agent was initially reported by Dennis [Dennis JW, Cancer Res 1986; 46: 5131-5136]. Subsequently, Seftor *et al,* [Melanoma Res 1981; 1: 43-54] reported that swainsonine induced changes in cell surface oligosaccharide composition and structure with a simultaneous downregulation of gelatinase-A (MMP-2) in two human malignant melanoma cell lines.

A standard cytotoxicity assay was used to optimise working concentrations of the drugs on seven human brain tumour-derived cell lines of various histological type and grade of malignancy. A qualitative assessement by gelatin zymography revealed that the effect of these agents varied between the seven cell lines such that the low grade pilocytic astrocytoma was unaffected by three of the agents. In contrast,

downregulation of the two gelatinases, MMP-2 and MMP-9 was seen in the grade 3 astrocytoma irrespective of which agent was used. Generally, swainsonine was the least effective whereas the citrus flavonoids, particularly nobiletin, showed the greatest downregulation of secretion of the MMPs. Furthermore, captopril and nobiletin were most efficient at inhibiting *in vitro* invasion, migration and adhesion in four representative cell lines (an ependymoma, a grade II oligoastrocytoma, an anaplastic astrocytoma and a glioblastoma multiforme). Yet again, the effects of the four agents varied between the four cell lines. Nobiletin was, nevertheless, the most effective agent used in these *in vitro* assays. In conclusion, the differential effects of these putative anti-invasive agents on each of the various parameters studied may be due to interference with MMPs and other mechanisms underlying the invasive phenotype. From these studies, it is clear that these agents, especially the citrus flavonoids, could be of significant therapeutic value.

The increased expression of MMP-2 and -9 in brain tumours *in vitro* is well documented [Rooprai *et al*, 1997, *ibid*]. For an agent to be of any therapeutic value for gliomas it should be able to down-regulate MMP expression. The data disclosed herein indicates that the four putative anti-invasive agents had a differential effect *in vitro* on MMP expression in seven cell lines derived from human brain tumours of various histological types and grade of malignancy. Tangeretin and nobiletin were generally effective at downregulating MMP-2 and MMP-9 expression in the cell lines studied, compared with swainsonine and captopril. In addition, the effects of the four agents varied considerably depending on the cell lines utilised. For example, MMP expression by the low-grade pilocytic astrocytoma cell line was generally unaffected by the four agents whereas both MMP-2 and MMP-9 expression by the high-grade glioma (IPMC-A3) was downregulated irrespective of the agent used.

Swainsonine is known to inhibit Golgi alpha-manosidase II, and appears to be least effective compared to the other three agents in the present study. Nevertheless its ability to down-regulate MMP expression is consistent with previous reports by Seftor *et al* [*ibid*] who pre-treated two malignant and invasive human melanoma cell lines

with it. They found that there was a correlation between the swainsonine-induced changes in the cell surface oligosaccharide composition with a concomitant decrease in the mRNA and secreted levels of MMP-2 and the ability of these cells to invade. Further studyies by Korczak and Dennis [Korczak B *et al*, Int J Cancer 1993; 53: 634-639] reported a 3-fold enhancement of the tissue inhibitors of metalloproteinases (TIMP) mRNA levels in murine mammary carcinoma cells cultured in the presence of swainsonine for 48 hours. It has been suggested that these effects influence the invasive phenotype of the cells and, since TIMPs are inhibitors of MMPs, it has been postulated that this may be a possible mechanism of action of swainsonine.

The inhibitory effect of captopril on MMP-2 and -9 expression confirms previous reports by Nakagawa *et al,*[*ibid*] and Sorbi *et al,* [Sorbi D *et al*, Kidney Int 44: 1266-1272]. Nakagawa *et al* showed that captopril inhibited invasion in a glioma cell line (T98G) *in vitro* assessed by gelatin zymography, whereas Sorbi *et al* demonstrated that captopril reduces MMP-2 and -9 activity in primary and secondary mesangial cell cultures in a dose-dependent manner. Both groups suggested that the captopril reversibly inhibits both MMPs by interacting with the zinc ion at their active sites.

From the results of this study, the citrus flavonoids seem to be most efficient at down-regulating the secretion of both MMP-2 and -9. However, nobiletin appears to be the best candidate for adjuvant therapy as it is more effective than tangeretin. A similar finding has been reported by Ito *et al,* [Annals N Y Acad Sci 1999; 878: 632-635] who examined the effects of nobiletin, tangeretin, sinensetin and their derivatives on the production of the latent forms of MMP-1, -3 and -9 in rabbit synovial fibroblasts. In particular, Ito *et al* found that nobiletin suppresses the production and gene expression of MMP-9 more effectively than that of MMP-1 and -3. In view of this, it is possible that the mechanisms involved in the action of flavonoids on MMP secretion are not only different from those of captopril and swainsonine but more complex as well. Indeed, the biological effects of flavonoids have been reported to occur mainly through their interaction with regulatory enzymes [Abou-Shoer M *et al*,

J Nat Prod 1993; 56: 967-9696; Constantinou A *et al*, J Nat Prod 1995; 58: 217-225; Laughton MJ *et al*, Biochem Pharmacol 1991; 42: 1673-1681].

Of the four compouns studied, swainsonine was generally observed to be one of the least effective compounds at inhibiting migration and invasion. Even so, migration and invasion were still inhibited to a significant extent in all four cell lines investigated. Swainsonine also has a marked effect on cell adhesion to collagen IV substrate. Collagen IV is a major constituent of endothelial basement membranes and it is believed to be is a ligand recognised by adhesion molecules on glioma cell membranes, since tumour cells are frequently reported to invade along the course of adjacent blood vessels.

The results of this study indicate that both tangeretin and nobiletin have the potential to retard motile or invasive behaviour, but that nobiletin is consistently more effective in this respect. Indeed, nobiletin has the greatest effect of the four agents tested. These two compounds are both effective at inhibiting MMP secretion and yet have widely different effects upon invasion. The reason why two apparently similar compounds have different efficacies, or indeed differentially affect the tested parameters, is undoubtedly related to their chemical structures. This in itself is remarkable since they differ by only a single methoxy group.

Of the four agents tested captopril was repeatedly shown to be one of the most efficacious at inhibiting *in vitro* invasion and migration. Only the flavonoid nobiletin showed a marginally greater overall effect. Captopril effectively inhibits adhesion, which would explain its anti-motile properties, but its effects upon MMP secretion varies between different cell lines. Undoubtedly these effects may, in part, be accounted for by the direct inhibition of MMPs by captopril. A study by Nakagawa *et al*, 1995 [Nakagawa *et al*, *ibid*] arrived at much the same conclusion: glioma cell invasion through the reconstituted ECM preparation Matrigel was inhibited by captopril, probably as a result of the latter's sequestration of Zn^{2+} ions. Indeed

Nakagawa *et al* found that addition of excess zinc to the culture medium could completely reverse the effects of captopril.

By way of summary, the studies disclosed herein have shown that swainsonine, captopril, tangeretin and nobiletin have differential effects on the various parameters of brain tumour invasion *in vitro*. Moreover, the effects were observed to vary between cell lines. The most effective agents at downregulating MMP expression were the citrus flavonoids, particularly nobiletin, whereas swainsonine was the least effective. Although, captopril and nobiletin were most efficient at inhibiting *in vitro* invasion, migration and adhesion, nobiletin was, nevertheless, the most effective agent. The differential effects seen, on the various parameters underlying brain tumour invasion, by these putative anti-invasive agents may be due to interference with MMP-2 and MMP-9 and other mechanisms underlying the invasive phenotype.

To conclude, the applicants have shown that naturally occurring flavonoids from fruits and vegetables may serve as therapeutic agents against a range of brain disorders. In particular, it has been shown that flavonoids may serve as potent anticancer agents in brain tumour therapy. This efficacy is based on the ability of the agents to interfere with the mechanisms controlling the invasion of brain tissue, for example adhesion, proteolysis and migration. As well as non-cytotoxic effects such as the downregulation of MMP-2 and –9, data also suggests that flavonoids have an additional effect in causing cell death. This dual effect of flavonoids in tumour cells makes them very promising candidates as targets for brain tumour therapy. In addition, the applicants have also demonstrated the activity of flavonoids against neurodegenerative disorders, particularly Parkinson's disease.

The present invention will now be described only by way of example and with reference to the accompanying figures, wherein:

Figure 1 shows the chemical structures of captopril, swainsonine, tangeretin and nobiletin.

Figure 2 shows the correlation between cell number and absorbance for a typical cell line (IPMC) as measured by MTT assay. Data represents mean ± SD. Six wells were used for each agent tested and the assay was repeated 3 times.

Figure 3 shows a typical zymogram for gelatinase-A (MMP-2, 72 kDa) and gelatinase-B (MMP-9, 92 kDa) showing the effects of four putative anti-invasive agents on an anaplastic astrocytoma (IPMC-A3). 1 = control, 2 = swainsonine (0.3mg/ml), 3 = captopril (30 mM), 4 = tangeretin (4g/ml), 5 = nobiletin (4μg/ml).

Figure 4 shows the effect of swainsonine (Swa), captopril (Cap), tangeretin (Tan), nobiletin (Nob) or control (Con) on *in vitro* migration, invasion and adhesion assays for ependymoma cell line, IPMA-E. Solid black columns represent migration data, open columns represent invasion data and hatched columns represent adhesion data. Data represents mean ± SD as a percentage of control value.

Figure 5 shows the effect of swainsonine (Swa), captopril (Cap), tangeretin (Tan), nobiletin (Nob) or control (Con) on *in vitro* migration, invasion and adhesion assays for oligo-astrocytoma cell line, IPSH-OA2. Solid black columns represent migration data, open columns represent invasion data and hatched columns represent adhesion data. Data represents mean ± SD as a percentage of control value.

Figure 6 shows the effect of swainsonine (Swa), captopril (Cap), tangeretin (Tan), nobiletin (Nob) or control (Con) on *in vitro* migration, invasion and adhesion assays for anaplastic astrocytoma cell line, IPMC-A3. Solid black columns represent migration data, open columns represent invasion data and hatched columns represent adhesion data. Data represents mean ± SD as a percentage of control value.

Figure 7 shows the effect of swainsonine (Swa), captopril (Cap), tangeretin (Tan), nobiletin (Nob) or control (Con) on *in vitro* migration, invasion and adhesion assays for glioblastoma multiforme cell line, AMH-FB. Solid black columns represent

migration data, open columns represent invasion data and hatched columns represent adhesion data. Data represents mean ± SD as a percentage of control value.

Figure 8 shows the results from a control study in which a glioblastoma multiforme cell line was left untreated and photographed every 5 minutes for 24 hours using time-lapse video microscopy (controls). Figures 8(a) and (b) represent photographs taken of the cell line by the video recorder initially and after 24 hours, respectively. The latter figure indicates tumour cell proliferation.

Figure 9 shows the effect of treating a glioblastoma multiforme cell line with chokeberry (100 μg/ml), photographed using time-lapse video microscopy. Figures 9(a) and (b) represent the effects after 30 min and 24 hours of treatment respectively.

Examples

Materials and Methods

The flavonoids used herein were prepared by a commercial extraction process and were obtained from the Florida Department of Citrus, Lake Alfred, FL 33850.

1. Studies on the distribution of tangeretin

Male Sprague-Dawley rats with starting weights 225±25 g were housed in groups of 3 with free access to food and water, under controlled temperature (21°C ± 1°C) and a 12 hour light/dark cycle (light on 07.00 hrs). All scientific procedures were carried out with the approval of the Home Office, U.K. Rats were administered via gavage 10 mg/kg of tangeretin (Department of Citrus, State of Florida, U.S.A) or drug vehicle (10% Cremophor, Sigma, U.K.) daily for 28 days (n=6 per group). Rats were killed by cervical dislocation followed by decapitation. Blood from the trunk was collected into EDTA tubes (Sarstedt, U.K.) and the plasma was separated by spinning the samples at 2,000 g for 20 min. The kidney, liver, spleen, lungs and heart were removed from each animal and immediately frozen on dry ice. The brain was removed from the skull onto a chilled platform and regionally dissected into the cerebellum, frontal cortex, cortex, hypothalamus, hippocampus, striatum and brain stem and immediately frozen on dry ice. All tissue samples were stored at -80°C until analysis.

Tangeretin was extracted from the weighed tissue samples/plasma by homogenizing the samples in 10 volumes of homogenizing medium (80% methanol with 100 µg/ml of the internal standard prednisolone; Lee YS *et al*, Pharmacol 1998, 56: 314-317) as described by Ameer *et al* [Clin Pharmacol Ther 1996, 60: 34-40]. The homogenates were incubated at 40°C for 10 min and subsequently centrifuged at 12,700 g for 10 min. The supernatant was removed and filtered through a 0.2 µm filter (Whatman, U.K.). HPLC analysis was performed using as isocratic pump (Dionex, U.K.) at a flow rate of 1 ml/min, an ODS column (5 µm, 4.6 mmx 15 cm Ace, U.K.) maintained at 40°C and connected to an UV detector (Holochrome, Gilson, U.K.). Chromatographic data were collected and analyzed by computer based software

(Chromeleon, Gynkotek, U.K.). The mobile phase consisted of 70% methanol, 30% water and 0.6% acetic acid (v/v) and detector set at 280 nm.

2. Studies of tangeretin on rat model of PD

Male Sprague-Dawley rats with starting weights 225±25 g were housed in groups of 3 with free access to food and water, under controlled temperature and a 12 hour light/dark cycle (light on 07.00 hrs). Rats were administered by gavage 20 mg/kg of tangeretin (Department of Citrus, State of Florida, U.S.A) or drug vehicle (10% Cremophor, Sigma, U.K.) daily for 4 days (n=6 per group). On the 4th day 1 hr after tangeretin or vehicle administration, rats were anaesthetized with Small animal Immobilon® (0.04 ml/rat, i.m.) and 6-hydroxydopamine (8 μg in 4 μl of 0.1% ascorbic acid/saline solution) was injected onto median forebrain bundle (stereotactic co-ordinates: 2.2mm anterior, +1.5 lateral from bregma and –7.9 ventral to dura with ear bars 5 mm below incisor bars; Paxinos G, Watson C (1986), ed 2. London, UK: Academic Press Ltd) as described by Datla *et al* [Mov Disord (in press)]. A 5 min period was allowed for drug equilibration. Post-operative care included individual caging and mashed diet.

One week after 6-hydroxydopamine lesions, rats were killed by cervical dislocation and the brains were dissected out immediately. A coronal section was made at the level of hypothalamus and fore brain and hind brain parts were separated. Hind brain was fixed for 7 days in 4% paraformaldehyde, followed by cryoprotected with 30% sucrose solution for 2-3 days.

From the fore brain left (lesioned side) and right striata (control side) were dissected out. The fixed tissue and the striata were stored at -80°C until further analysis.

(a) Tyrosine hydroxylase immunohistochemistry

TH immunocytochemistry was carried out as described by Dexter *et al* [Neuroreport 1994, 5: 1773-1776]. Briefly, free floating sections were incubated in 20% normal goat serum (NGS) for 60 min at room temperature and rinsed twice in a solution of 0.1 M

PBS containing 1% NGS and 0.05% Triton-X100. They were then incubated with the primary antibody, rabbit anti-TH (Chemicon, UK), diluted to 1 in 3000 with PBS plus 1% NGS, overnight at room temperature. The sections were rinsed twice in PBS and incubated with the second antibody, biotinylated anti-rabbit IgG from an ABC Vectastain kit (Vector Lab, U.K.) for 60 min and an avidin/biotin complex was formed. The TH immunocomplex was visualised by pre-incubating sections with 0.05% diaminobenzidine (DAB) in Tris buffer for 5 min. Hydrogen peroxide (0.01%) was added to the DAB solution and the incubation extended for a further 1 min. The sections were mounted onto slides, air dried and dehydrated through 70%, 96% and 100% ethanol followed by Histoclear®. Dopaminergic cells having robust TH immunoreativity were then examined under a microscope (Nikon Eclipse E800), images were captured by a Xillix CCD digital camera and automated counting of immunopositive cells was then performed using Image Proplus software (Datacell, U.K.). In the region B (as described by Carman LS *et al*, Brain Res 1991, 553: 275-283) each TH+ neurons were counted on the control side and compared with the lesioned side.

(b) Strital monoamine estimations by HPLC analysis

Monoamines were extracted essentially as described by Sullivan *et al* [Eur J Neurosci 1998, 10: 57-63] and in brief, striata were individually weighed and homogenized in 0.5 ml of ice-cold buffer (50 mM trichloroacetic acid, 0.5 mM, disodium ethylenediaminetetraacetic acid (EDTA), 0.5 pmol/l,3,4-dihydroxybenzylamine hydrobromide as an internal standard) for 30 sec using ultrasonicator (Soniprep 150, Sanyo,U.K.). After keeping on ice for 10 min the samples were centrifuged (Heraeus, U.K.) at 12000 g for 10 min, 4°C. Supernatants were filtered (0.2 μm, Whatman, U.K.) and loaded onto an autosampler (Gina 50, Gynkotek, U.K.) kept at 5°C on-line with HPLC system. Samples were analyzed for dopamine using phosphate buffer (0.1 m KH_2PO_4, 0.1 mM EDTA, 1 mM octyl sodium sulfonate, 10 % methanol V/V, pH 2.75 adjusted with orthophosphoric acid; flow rate 0.9 ml/min), Altex 3μm ODS column (4.6 mm x 7.5 cm, Beckman, U.K.), analytical cell (model 5011, ESA Analytical, U.K.) and Coulochem-II detector with electrode one set at -0.20 V and electrode two at +0.34 V

with respect to palladium reference electrode (ESA Analytical, U.K.) as described by Datla *et al* [Neuropharmacol 1996, 35: 315-320]. Chromatographic data were collected and analyzed by computer based software (Chromeleon, Gynkotek, U.K.). Both control and tangeretin treated groups from lesioned and unlesioned sides were analyzed on the same day.

Results

In the vehicle treated rats there was no detectable amounts of Tangeretin in the plasma, peripheral organs or the brain (data not shown). After 28 days of oral tangeretin, highest concentrations were found in the brain, but also detectable in all regions most peripheral organs and the plasma. Brain levels were for e.g. about 6 times higher than peripheral organs (Table 1). Within the brain, concentrations of tangeretin showed considerable variation, with the brain stem and cerebellum having the lowest concentrations (0.17 and 0.27 ng/mg tissue respectively) whilst the highest concentrations were seen in the hippocampus, striatum and hypothalamus (2.0, 2.36 and 3.88 ng/mg tissue respectively). In the periphery, the liver had the highest concentration, while the heart and lung contained similar concentrations to the plasma, and the kidney contained the lowest concentrations (Table 1).

The number of tyrosine hydroxylase positive (TH+) neurons on the control side brain vehicle treated group is comparable to the tangeretin treated group (20 mg/kg x 4 days; p.o.) at the level B. On the lesioned side of the vehicle treated rats there was about 56% loss of TH+ neurons with 6-Hydroxydopamine (6-OHDA; 8 μg). On the other hand, only about 17% loss of TH+ neurons were seen in tangeretin treated rats (Table 2).

Striatal dopamnine levels on the control side were comparable in both vehicle and tangeretin treated rats. However, lesioning with 6-OHDA decreased dopamine levels by 55% in the vehicle treated group. In tangeretin pre-treated group, 6-OHDA induced dopamine loss was only 29% (Table 3).

3. Investigating the Effects on Brain Cancer

Initially all cell lines were examined for MMP secretion by zymography and then four of these cell lines were selected to represent each histological type and grade of malignancy for *in vitro* motility, adhesion and invasion assays.

Cell cultures

Surgical samples from patients with various types of brain tumours were obtained from the Neurosurgical staff at King's College and Atkinson Morleys' Hospitals, London. Biopsy material was used to establish seven short-term cell cultures. All the samples analysed were diagnosed by a neuropathologist, according to the World Health Organisation criteria [Kleihues P *et al,* WHO Classification, Second Edition, 1993; Berlin, Springer Verlag]. The samples chosen in this study included one ependymoma, one pilocytic astrocytoma, one grade 2 oligoastrocytoma, one grade 3 anaplastic oligoastrocytoma, one grade 3 astrocytoma and 2 glioblastoma multiforme (Table 4). A variety of brain tumours were chosen in order to identify which type of tumour was most sensitive to the effects of the agents under investigation.

Maintenance of Cell Cultures

Cells were routinely propagated as monolayers in Dulbecco's Modified Eagle's Medium (DMEM)(Life Technologies) supplemented with 10% heat-inactivated foetal calf serum (Sigma) and 1% antibiotic/antimycotic solution (penicillin/streptomycin/ amphotericin, Sigma); this is referred to as complete medium. The buffer was maintained at physiological pH by equilibration with 5% CO_2 in air in a standard humidified incubator at 37°C. For serum-free conditions, a serum free medium (SFM) was formulated from Dulbecco's MEM F-12 (Ham) nutrient mix (Life Technologies) supplemented with progesterone at 10nM (Sigma), 1% antibiotic-antimycotic solution, tri-iodo thyronine (1ng/ml) (Sigma), and 0.5% insulin-transferrin-sodium selenite media supplement solution (Sigma).

MTT Assay for Cell Viability and Determination of IC_{50}

Since it is known that certain anti-cancer agents induce apoptosis in human cell lines a chemosensitivity assay was performed to obtain a drug-dose response, which was assessed with the MTT assay. Time-lapse video microscopy was used to study the effects of the flavonoids (at the chosen concentrations) on migration/proliferation of monolayers of brain tumour cell lines.

The MTT assay is based on the ability of the mitochondrial enzyme succinate dehydrogenase to reduce the tetrazolium salt, MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan. The tetrazolium salt MTT works as an indicator of mammalian cells survival and proliferation [Marshall N *et al*, Growth Regulation 1995; 5: 69-84]. The assay detects living cells only and is used to measure cytotoxicity and proliferation. The results are analysed on a multi-well scanning spectrophotometer and show a high degree of precision.

Briefly, 20 µl of 9% Triton X 100 in phosphate buffer saline (PBS) was placed in a single row of wells in a 96 well plate, incubated for 45 min and used as a background reading. After appropriate treatments, cells were incubated for 2 hours in MTT (1 mg/ml) in DMEM without phenol red indicator. This MTT solution was removed and the remaining dark blue crystals were dissolved in 200 µl dimethyl sulphoxide (DMSO, Sigma). Complete dissolution of the crystals was achieved by shaking the plate gently for 10 min at room temperature. Plates were read on a Dynatech plate reader at a wavelength of 570 nm with a 15-sec mixing time. Plates were always read within 30 minutes of adding the DMSO.

The optimal cell number for chemosensitivity testing was estimated to be 10,000 cells per ml for all cell lines utilised and this plate density was subsequently used to determine the IC_{50} for the agents under study. Cells from the seven cell lines were plated into 96 well plates in 200µl DMEM containing no foetal calf serum or phenol red indicator. Cells were left overnight to adhere after which the medium was replaced with fresh medium containing a range of concentrations of the test

compound (swainsonine (Sigma UK): 3ng-3μg; captopril (Sigma UK): 10ng-10μg; tangeretin and nobiletin (Department of Citrus, Florida): 10ng-10μg) and incubated for 24 hours at 37°C. Six wells were used for each cell line for a given agent and repeated three times. Solutions containing the anti-invasive agents were subsequently removed and each well incubated with 100μl MTT solution (1mg/ml) for two hours. The plates were read as described above.

Zymography

Cells were routinely propagated in culture and as confluence approached were weaned down from 10% serum-supplemented medium to serum free medium (SFM) over a 48-hour period. They were then treated with the chosen concentrations of anti-invasive agents: swainsonine at 0.3 μg/ml medium in 1% ethanol, captopril at 30 μg/ml, tangeretin and nobiletin at 4μg/ml in dimethyl sulphoxide (DMSO). Control samples were incubated in serum-free medium with DMSO or ethanol and maintained for 48 hours where upon they were harvested and counted and the medium collected. These cell-conditioned medium samples served as the source of MMP-2 and MMP-9 for zymography. They were freeze-dried and reconstituted in an appropriate volume of sterile water according to the number of cells in the monolayer from which they had been harvested to provide a standard of 1×10^6 cells/ml of reconstituted volume.

Zymogram analysis was performed by a modification of the methods of Heussen and Dowdle [Anal Biochem 1980; 102: 196-202] and Rucklidge *et al,* [Biochem Biophys Res Comm 1990; 172: 544-550] to investigate the activities of the two gelatinases: MMP-2 and MMP-9. Briefly, the substrate gelatin (with a final concentration of 1 mg/ml) was co-polymerised into an 11% acrylamide resolving gel at the time of gel casting. An aliquot (40 μl) of cell-conditioned media was mixed with 15μl of a loaded buffer (tris base) containing 30% glycerol, 7.7% sodium dodecyl sulphate (SDS) and 0.3% bromophenol blue at pH 6.8 before being loaded into stacking gel wells. Following electrophoresis in a Mini Protean II Cell (Bio Rad), the gels were immersed in Triton X-100 (2.5%) for 1 hour to remove the SDS. The enzymes were then reactivated by incubating the gels in a 50 mM Trizma-HCl buffer (pH7.6)

containing 10 mM $CaCl_2$ at 37^0 C for 24 hours before being stained overnight with 0.075% Coomassie Blue in methanol: acetic acid:water (10:1:10 v:v:v). Finally, the gels were de-stained with methanol; acetic acid; water (2:1:10 v:v:v) for 24 hours to reveal clear bands containing proteolytic activity on a dark blue background. The bands were identified in terms of their molecular weight: 92 kDa and 72 kDa, corresponding to MMP-9 and MMP-2, respectively. Proteolytic activity was evaluated using an IBAS 200 image analyser and verification of the MMP status of enzymes detected was achieved by substituting the 10 mM $CaCl_2$ with an MMP inhibitor, 1,10-phenanthroline.

Cell Migration Assays

In-vitro migration in response to different reagents was monitored by a chemotaxis assay using 12-well Boyden transwell units incorporating 12 μm porosity polycarbonate filters (Costar, Cambridge, U.K.). Having weaned down cells from serum containing to SFM, the cells were harvested counted and re-suspended in SFM in the presence or absence of the anti-invasive agents. Cell suspension (200μl), containing around 10,000 cells, were placed in the upper compartment of the transwell unit. Each lower compartment of the transwell contained SFM containing platelet derived growth factor (PDGF) AB variant (Sigma, UK) at 10ng/ml that served as a chemoattractant. After 48 h incubation at 37°C in a humidified 95% air, 5% carbon dioxide atmosphere, cells were fixed with acetic acid/alcohol and stained with Giemsa. Cells on the upper surface of the filter were removed by wiping with a cotton swab and motility was determined by counting the cells that had migrated to the lower side of the filter using a phase contrast microscope at 100 × magnification. Four filters were seeded per cell sample being tested and ten field counts were performed per filter. Each sample was assayed in triplicate and repeated on two separate occasions.

Invasion Assay

In vitro invasiveness was measured by the method of Albini *et al* [Cancer Res 1987; 47: 3239-3245] with modifications using transwell chambers described above except that the upper surfaces of the filters were precoated with extracellular matrix proteins

prior to the assay. The protein used was human placental collagen type IV (10μg/ml, Sigma). Solutions of collagen type IV were air-dried onto the upper surfaces of the polycarbonate filters overnight. Prior to addition of the cells, excess medium was removed from the upper compartment. All assays were carried out in triplicate and repeated on two separate occasions.

MTT Adhesion Assay

The MTT adhesion assay was a modification of the method described previously [Rooprai HK *et al*, Int J Devl Neurosci 1999; 17: 613-623]. Briefly, 96-well plates were coated overnight with collagen type IV protein as described above. Any remaining protein solutions were removed with a single flick of the plate and the wells thrice washed with phosphate buffered saline (PBS). Cells were weaned down to SFM, harvested, plated (10,000 cells per well in 200 μl SFM) and incubated for 2h at 37°C. In some wells the different agents were added prior to plating for assessment of their effect. Bovine serum albumin-coated surfaces were used as controls. After this time, non adherent cells were removed from the wells with a single flick of the plate and the wells carefully rinsed with PBS. Each assay was repeated in triplicate. The MTT assay was subsequently performed as described as above.

Statistical Analysis

The data was analysed statistically using analysis of variance tests (ANOVA) to measure the significant difference between the means using the Tukey-HSD test with statistical significance set at $P < 0.05$.

Results - Brain Tumour Cell Lines

Time-lapse Videomicroscopy

The results indicate from time-lapse videomicroscopy studies show that there is a differential effect of the flavonoids from berries (elderberry, chokeberry, bilberry) on malignant brain tumours compared to the citrus flavonoids.

Typical results are illustrated in Figures 8 and 9.The results from a control study in which a glioblastoma multiforme cell line was left untreated and photographed every 5 minutes for 24 hours are shown in Figure 8. Figures 8(a) and (b) represent photographs taken of the cell line by the video recorder initially and after 24 hours, respectively. The latter figure indicates tumour cells proliferation. Figures 9(a) and (b) represent the effects after 30 min and 24 hours of treatment with chokeberry (100 μg/ml), respectively, on a glioblastoma multiforme cell line. Although the tumour cells have already rounded after half an hour of treatment (Figure 9a) compared to the untreated cells (Figure 8a) the effect is more drastic after prolonged treatment (24 hours) since the cells are rounded and detached completely from the plate (i.e. cell death) (Figure 9b).

MTT assay for cell viability and IC_{50}

The results from the MTT assay demonstrated a good correlation ($P < 0.05$) between cell viability and formazan production over an incubation period of 48 hours (Data not shown). The IC_{50} for the four anti-invasive agents were estimated to be 0.3 μg/ml for swainsonine, 30 μg/ml for captopril, and 4 μg/ml for tangeretin and nobiletin in all seven cell lines studied. The optimum period of incubation was 48 hours and the optimal cell number for chemosensitivity testing was estimated to be 10,000 cells/well in a 96 well plate. Figure 2 shows correlation between cell number and absorbance at 570 nm for a representative cell line (IPMC).

Zymography

The controls using either DMSO or ethanol showed no effect on MMP secretion. The cumulative results for the qualitative analysis of MMP-2 and MMP-9 secretion in 7 brain tumour cell lines under the influence of various agents, are represented in Table 5. Figure 3 represents a typical gelatin zymogram. Proteolytic activity is indicated by the presence of clear bands on a dark background and the intensity of the band is proportional to the enzyme expression. All the untreated cell lines showed presence of both MMP-2 and -9 with the exception of two high-grade cell lines (IPAB-AO3 and IPLC-GM) which showed no expression of MMP-9.

The zymogram results (Table 5) showed that the four agents had a differential effect upon MMP secretion by the tumour cell lines. For an agent to be of any therapeutic value it should be able to down-regulate MMP expression. Taking each agent into consideration, it appears that swainsonine was least effective as it showed down-regulation of MMP-2 in 3 high grade cell lines (IPAB-AO3, IPMC-A3 and IPLC-GM) and down-regulation of MMP-9 in only one cell line (IPMC-A3). In the other cell lines, swainsonine had no effect on enzyme secretion or caused an increase in enzyme secretion. Captopril showed down-regulation of both MMP enzymes in two low-grade tumour cell lines (IPMA-E and IPSF-PA) and one high grade glioma cell line (IPMC-A3). In the other cell lines, captopril had no effect on enzyme secretion or caused an increase in enzyme secretion. Interestingly, the citrus flavonoids, tangeretin and nobiletin were most effective, down-regulating MMP activity in most of the cell lines. Indeed, nobiletin was the best putative anti-invasive agent studied here as it down-regulated MMP expression in all but one cell line (IPSF-PA), a low grade, benign neoplasm (Figure 3).

Taking individual cell lines into consideration also reveals a differential pattern in that MMP expression by the low-grade pilocytic astrocytoma (IPSF-PA) was generally unaffected by the agents, with one exception: captopril down-regulated both MMP-2 and MMP-9 expression by this cell line. Furthermore, the MMP expression in the non-invasive ependymoma (IPMA-E) was downregulated, not only with captopril but nobiletin as well. The grade 2 oligoastrocytoma (IPSH-OA2) generally showed down-regulation of MMP-2 with all the agents except captopril, which was ineffective. In contrast, enzyme expression by the anaplastic astrocytoma (IPMC-A3) was downregulated to varying extents by all four agents investigated in this study. The four agents had differential effects on the two glioblastomas (AMHFB-GM and IPLC-GM) in that MMP-2 and -9 expression was downregulated by tangeretin and nobiletin only, whereas MMP-2 was downregulated by swainsonine and nobiletin. Moreover, in two of the cell lines (IPAB-AO3 and IPLC-GM) which showed no expression of MMP-9 in the controls, it was not surprising to see no change in its expression under the influence of the anti-invasive agents. MMP up-regulation was sometimes seen in

the different cell lines with swainsonine (AMHFB-GM) and captopril (IPSH-OA2, IPLC-GM and AMHFB-GM).

In vitro motility, adhesion and invasion assays

In the ependymoma cell-line IPMA-E; nobiletin and captopril had similar effects, down-regulating migration and adhesion to a similar degree (approximately 90 and 80% respectively, $P < 0.05$) and having a less profound but significant ($P < 0.05$) effect upon invasion (Figure4). Swainsonine and tangeretin both inhibited migration and invasion to a similar degree (approximately 70%), however swainsonine had a greater inhibitory effect than tangeretin upon adhesion (85% and 70% respectively).

In the IPSH-OA2 oligoastrocytoma cells, nobiletin and captopril produced the highest degree of inhibition, captopril having the more significant ($P < 0.01$) effect on migration and invasion (80-90% reduction in both cases) whereas, nobiletin has an effect of similar magnitude on invasion and adhesion (Fig 5). Swainsonine produced a smaller but significant effect, reducing migration and invasion (60%, $P < 0.05$), and adhesion (80%, $P < 0.01$). By contrast, tangeretin had no significant effects upon either migration or adhesion but did reduce invasion by around 70% ($P < 0.05$).

For the IPMC-A3 anaplastic astrocytoma cells, nobiletin and captopril were the most effective agents, reducing migration and invasion to a similar degree (85%, $P < 0.01$) but having slightly different effects on adhesion; nobiletin causing an 80-90% reduction ($P < 0.01$) and captopril around 70% ($P < 0.05$). Tangeretin and swainsonine overall had less profound but nevertheless significant ($P < 0.05$) inhibitory effects on all three parameters (Figure 6).

The data for the glioblastoma multiforme line, AMH-FB is given in Figure 7. Nobiletin and captopril are again the most efficacious compounds, both causing a significant ($P < 0.01$) reduction on all three parameters, the values ranging between 80-90%. Captopril produced a smaller reduction in adhesion by 65% ($P < 0.05$). Swaisonine and tangeretin both significantly decrease migration and invasion ($P <$

0.05) but have different effects on adhesion; swainsonine being a powerful inhibitor ($P < 0.01$) whereas tangeretin has no significant effect.

Various modifications and variations of the described methods of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the relevant fields are intended to be covered by the present invention.

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Table 1: Values represent ng/mg of tissue given as mean ± SEM; n=6 per group. * ng/ml of plasma. nd= not detectable.

	Tangeretin
peripheral tissue	
heart	0.12 ± 0.02
kidneys	0.05 ± 0.01
liver	0.59 ± 0.09
lungs	0.14 ± 0.01
spleen	nd
plasma*	0.11 ± 0.02
brain regions	
striatum	2.36 ± 0.25
hippocampus	2.00 ± 0.23
cerebellum	0.27 ± 0.05
hypothalamus	3.88 ± 0.48
cortex	0.46 ± 0.01
brain stem	0.17 ± 0.05
frontal cortex	0.67 ± 0.09

Table 2: Effects of tangeretin (20 mg/kg p.o. x 4) on tyrosine hydroxylase positive cells in the substantia nigra (level B; see methods and materials).

	control side	lesioned side	% decrease
Vehicle	168 ± 21	64 ± 12*	56 ±10
tangeretin	163 ± 7	135 ± 4[a]	17 ± 5[b]

Values represent mean ± SEM of tyrosine hydroxylase positive cells. N=6 per group. * $P<0.05$ vs. control side after paired Student's t-test. [a] $P<0.005$ vs. lesioned side of vehicle treated group, unpaired Student's t-test. [b] $P<0.05$ vs. vehicle group after unpaired Student's t-test. MANOVA comparisons with lesion as within subject factor and tangeretin as between subject factor revealed that there was effect of lesion ($F_{1,7}=11.658$; $P<0.02$) and effect of tangeretin ($F_{1,7}=7.335$; $P=0.03$).

Table 3: Effects of tangeretin (20 mg/kg, p.o. x 4 days) on striatal dopamine levels.

	control side	lesioned side	% decrease
Vehicle	1.05 ± 0.03	0.47 ± 0.11*	54.4 ± 10.5
tangeretin	1.10 ± 0.04	0.77 ± 0.09[a]	28.3 ± 5.20

Values represent mean ± SEM of dopamine levels given as ng/mg wet tissue. n=6 per group. * P< vs. control side after paired Student's t-test. MANOVA analysis with lesion as with in subject factor and tangeretin treatment as between subject factor revealed that there is effect of lesion ($F_{1,20}$ =33.2 P<0.001) and tangeretin ($F_{1,20}$ =5.11 P<0.05).

Table 4: Cell lines used in the study.

Cell line designation	Histological type and grade
IPMA-E	Ependymoma
IPSF-PA	Grade 1 Pilocytic astrocytoma
IPSH-OA2	Grade 2 Oligoastrocytoma
IPAB-AO3	Grade 3 Anaplastic oligoastrocytoma
IPMC-A3	Grade 3 Astrocytoma
AMHFB-GM	Grade 4 Glioblastoma multiforme
IPLC-GM	Grade 4 Glioblastoma multiforme

The prefix "IP" for each cell line designation refers to the Institute of Psychiatry, whereas "AMH" refers to the Atkinson Morleys' Hospital, London.

Table 5: Qualitative evaluation of MMP-2 and MMP-9 in 7 human brain tumour cell lines *in vitro*.

Cell line	Control		Swainsonine		Captopril		Tangeretin		Nobiletin	
	MMP-2	MMP-9	MMP-2	MMP-9	MMP-2	MMP-9	MMP-2	MMP-9	MMP-2	MMP-9
IPMA-E	+	+	NC	NC	↓	↓	↑	NC	↓	↓
IPSF-PA	+	+	NC	NC	↓	↓	NC	NC	NC	NC
IPSH-OA2	+	+	↑↑	↑↑	↑	↑	↓	↓	↓	↓
IPAB-AO3	+	-	↓	NC	NC	NC	↓	NC	↓	NC
IPMC-A3	+	+	↓↓	↓	↓	↓	↓↓	↓	↓↓	↓↓
AMHFB-GM	+	+	↑	↑	↑	↑	↓	↓	↓	↓
IPLC-GM	+	-	↓	NC	↑	NC	↑	NC	↓	NC

+ = presence of MMP secretion

- = absence of MMP secretion

NC = no change in enzyme secretion

↑ = upregulation

↓ = downregulation

Claims

1. A pharmaceutical composition for use (or when in use) in the treatment of brain disorders, said composition comprising one or more flavonoids admixed with a pharmaceutically acceptable diluent, excipient or carrier.

2. A pharmaceutical composition according to claim 1 wherein the flavonoid is capable of crossing the blood-brain barrier.

3 A pharmaceutical composition according to claim 1 or claim 2 wherein said brain disorder is a neurodegenerative disorder.

4. A pharmaceutical composition according to claim 3 wherein said neurodegenerative disorder is Parkinson's disease.

5. A pharmaceutical composition according to claim 1 or claim 2 wherein said brain disorder is a tumour.

6. A pharmaceutical composition according to any preceding claim wherein the flavonoid is derived from one or more of the following: a berry fruit, a citrus fruit, turmeric, green tea, grapes, grapeseed and soya.

7. A pharmaceutical composition according to claim 6 wherein the flavonoid is derived from a berry fruit, preferably elderberry, chokeberry or bilberry.

8. A pharmaceutical composition according to claim 6 wherein the flavonoid is derived from a citrus fruit.

9. A pharmaceutical composition according to claim 8 wherein said flavonoid is selected from tangeretin and nobiletin.

10. A pharmaceutical composition according to claim 6 wherein said flavonoid is derived from soya, and is preferably daidzein.

11. A pharmaceutical composition according to claim 6 wherein said flavonoid is derived from grapeseed, and is preferably proanthocyanidin.

12. Use of one or more flavonoids in the preparation of a medicament for treating brain disorders.

13. Use according to claim 12 wherein the flavonoid is capable of crossing the blood-brain barrier.

14. Use according to claim 12 or claim 13 wherein said brain disorder is a neurodegenerative disorder.

15. Use according to claim 14 wherein said neurodegenerative disorder is Parkinson's disease.

16. Use according to claim 12 or claim 13 wherein said brain disorder is a tumour.

17. Use according to any one of claims 12 to 16 wherein the flavonoid is derived from one or more of the following: a berry fruit, a citrus fruit, turmeric, green tea, grapes, grapeseed and soya.

18. Use according to claim 17 wherein the flavonoid is derived from a berry fruit.

19. Use according to claim 18 wherein the flavonoid is derived from elderberry, chokeberry or bilberry.

20. Use according to claim 17 wherein the flavonoid is derived from a citrus fruit.

21. Use according to claim 20 wherein said flavonoid is tangeretin.

22. Use according to claim 20 wherein said flavonoid is nobiletin.

23. A process for the preparation of a pharmaceutical composition according to any one of claims 1 to 11, said process comprising admixing one or more flavonoids with a pharmaceutically acceptable diluent, excipient or carrier.

24. A method of treating a brain disorder, said method comprising administering to a subject in need of treatment a therapeutically effective amount of one or more flavonoids.

25. A method of treating a brain disorder, said method comprising administering to a subject in need of treatment a pharmaceutical composition as defined in any one of claims 1 to 11.

26. A method according to claim 24 or claim 25 wherein the brain disorder is a neurodegenerative disorder.

27. A method according to claim 24 or claim 25 wherein the brain disorder is a tumour.

28. A pharmaceutical pack comprising one or more compartments, wherein at least one compartment comprises one or more flavonoids.

29. An assay method for identifying an agent that can directly or indirectly downregulate the expression of MMP-2 and/or MMP-9 in order to treat brain

disorders, preferably brain tumours, the assay method comprising: contacting an agent with MMP-2 and/or MMP-9; and measuring the activity of MMP-2 and/or MMP-9; wherein a downregulation of expression of MMP-2 and/or MMP-9 in the presence of the agent is indicative that the agent may be useful in the treatment of brain disorders, preferably brain tumours.

30. A process comprising the steps of:

(a) performing the assay according to claim 29;

(b) identifying one or more agents that can directly or indirectly downregulate the expression of MMP-2 and/or MMP-9; and

(c) preparing a quantity of those one or more identified agents.

31. A method of treating brain disorders, by downregulating expression of MMP-2 and/or MMP-9 *in vivo* with an agent;
wherein the agent is capable of directly or indirectly downregulating expression of MMP-2 and/or MMP-9 in an *in vitro* assay method;
wherein the *in vitro* assay method is the assay method defined in claim 29.

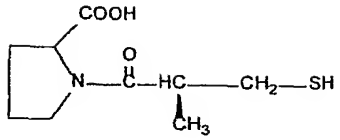
32. Use of an agent in the preparation of a pharmaceutical composition for the treatment of brain disorders, preferably brain tumours, wherein the agent is capable of directly or indirectly downregulating expression of MMP-2 and/or MMP-9 when assayed *in vitro* by the assay method according to claim 29.

33. An agent identified by the assay method according to claim 29.

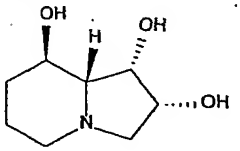
34. An agent according to claim 33 for use in medicine.

35. An agent according to claim 34 for use in treating brain cancer.

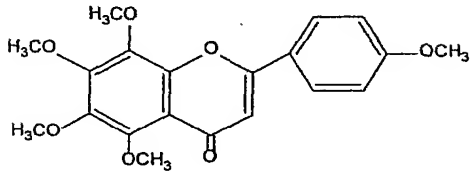
36. Use of one or more flavonoids in an assay for identifying candidate compounds that are capable of influencing the expression of MMP-2 and/or MMP-9.



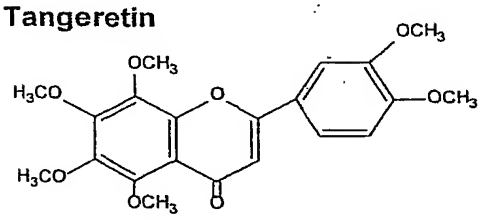
Captopril



Swainsonine



Tangeretin



Nobiletin

Figure 1

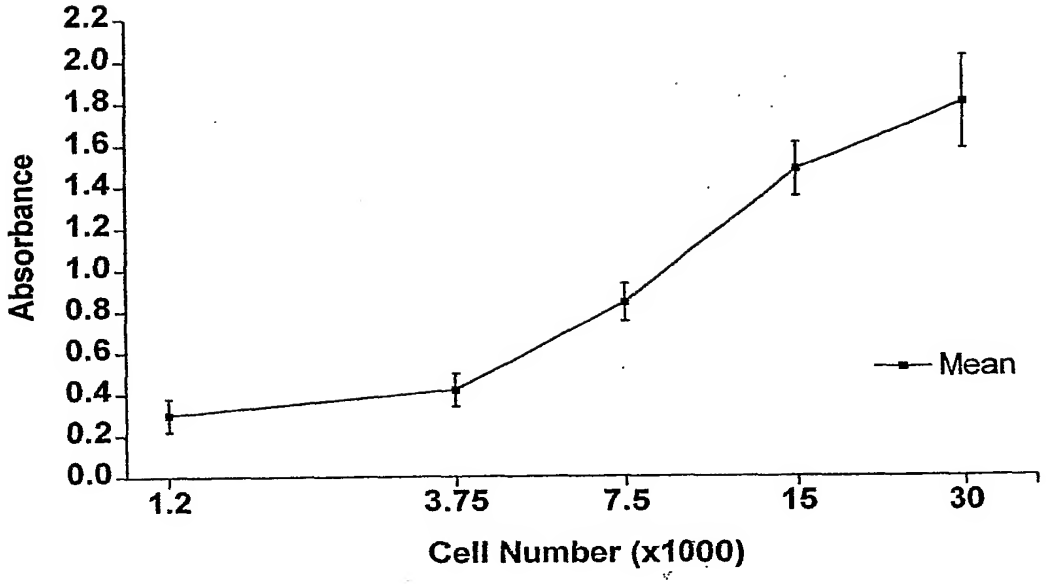

Absorbance
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1.2
3.75
7.5
15
30
Cell Number (x1000)
Mean

Figure 2

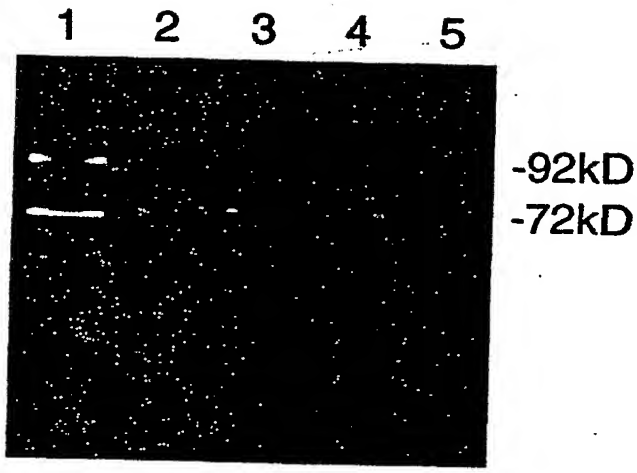


Figure 3

(4/8)

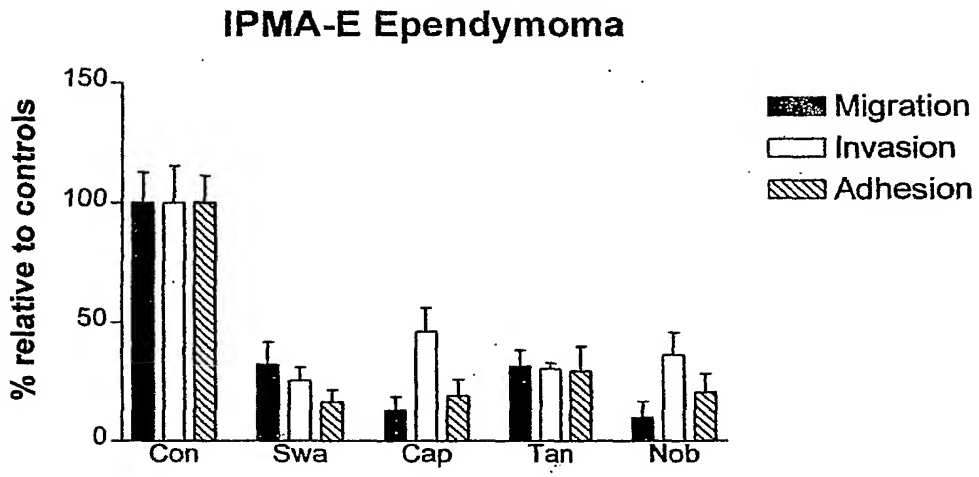

IPMA-E Ependymoma
% relative to controls
150
100
50
0
Con
Swa
Cap
Tan
Nob
Migration
Invasion
Adhesion

Figure 4

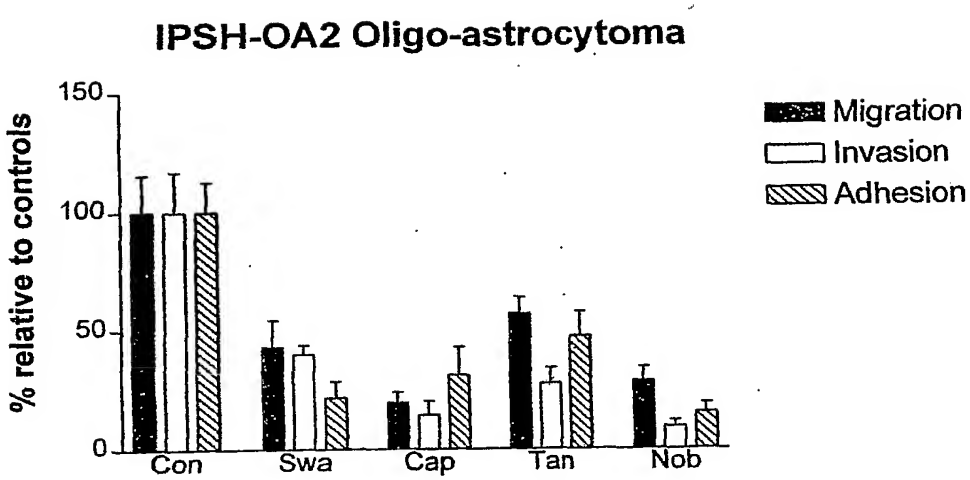

IPSH-OA2 Oligo-astrocytoma
% relative to controls
150
100
50
0
Con
Swa
Cap
Tan
Nob
Migration
Invasion
Adhesion

Figure 5

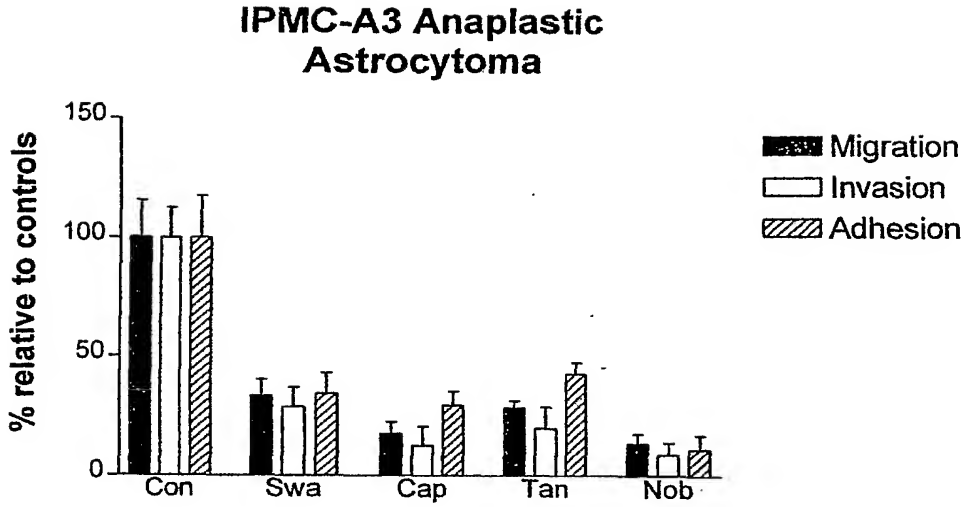

IPMC-A3 Anaplastic Astrocytoma
% relative to controls
150
100
50
0
Con
Swa
Cap
Tan
Nob
Migration
Invasion
Adhesion

Figure 6

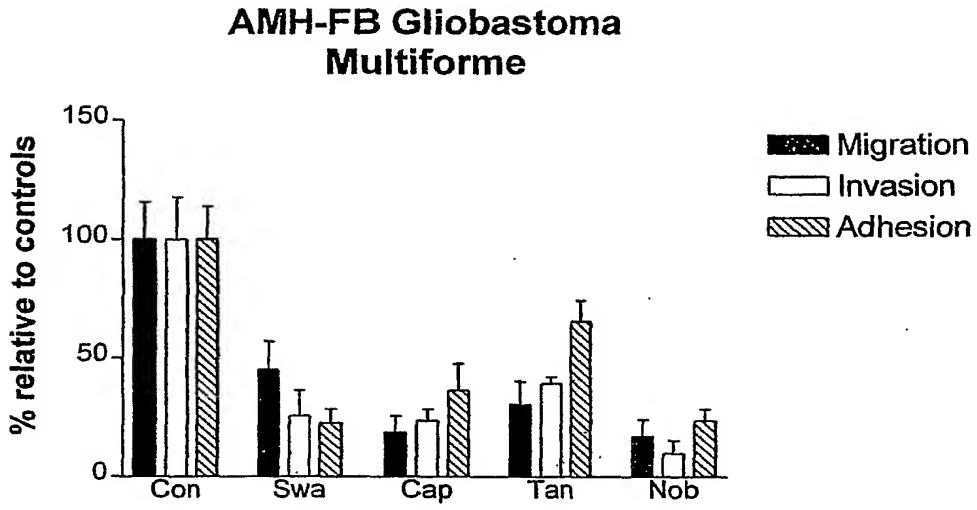

AMH-FB Gliobastoma Multiforme
% relative to controls
150
100
50
0
Con
Swa
Cap
Tan
Nob
Migration
Invasion
Adhesion

Figure 7

Figure 8

(a) **(b)**

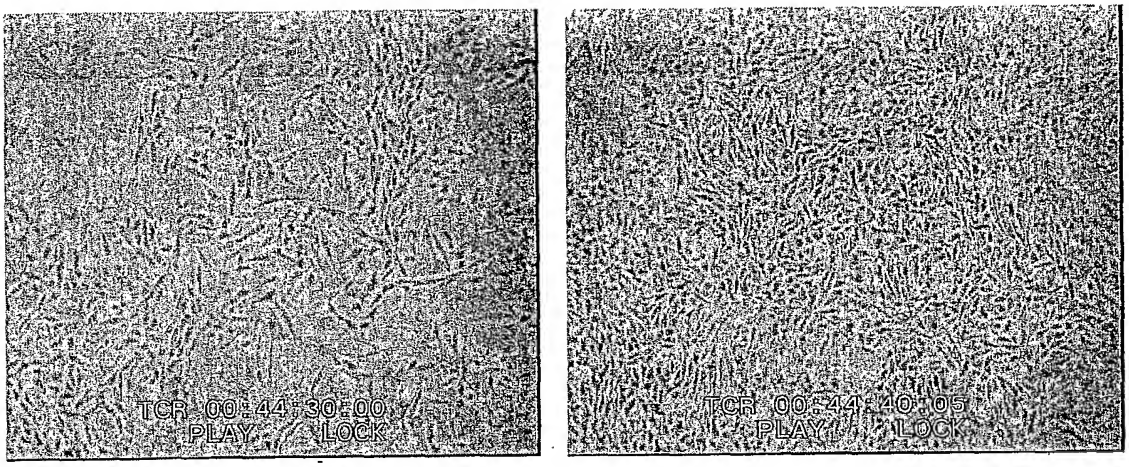
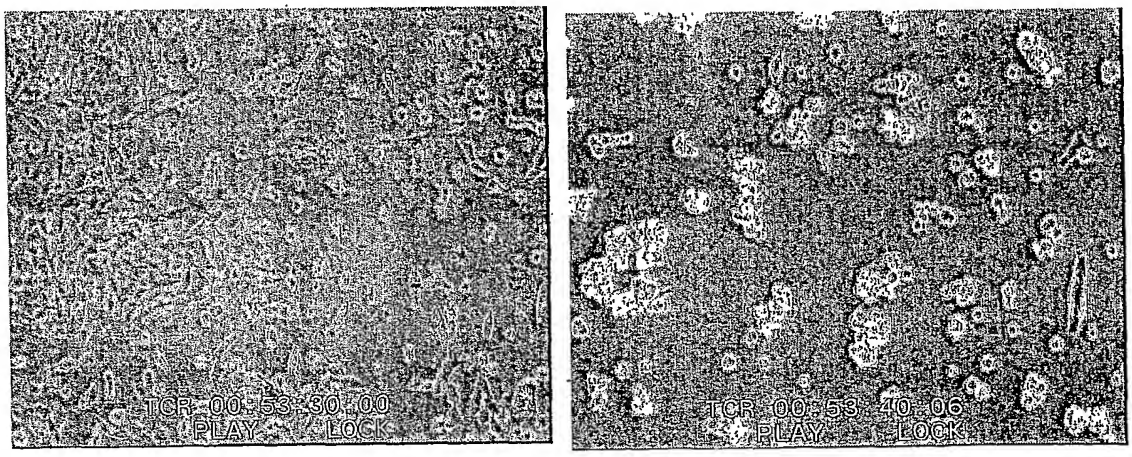


Figure 9

(a) **(b)**



INTERNATIONAL SEARCH REPORT

Inter nal Application No
PC1/GB 01/00056

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/35 A61P25/16 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, EPO-Internal, BIOSIS, EMBASE, MEDLINE, CHEM ABS Data, PHARMAPROJECTS .

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 004 558 A (HUANG LI JIU ET AL) 21 December 1999 (1999-12-21) *cf. abstract,col.2, lines 14-48, col. 7, lines 23-43 and lines 48-57*	1-28
X	WO 99 43335 A (NOVOGEN RES PTY LTD ;HUANG LI JUI (AU); THURN MICHAEL JOSEPH (AU)) 2 September 1999 (1999-09-02) *cf. abstract, page 1, 1st para., page 3, line 29 bridging with page 4, 1st para., claim 11*	1-28
X	US 5 733 926 A (GORBACH SHERWOOD L) 31 March 1998 (1998-03-31) *cf. abstract, col. 1, lines 33-60, col. 2, claim 1*	1-28

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 January 2002	05.04.02

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Stoltner, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 01/00056

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 436 129 A (MONTANA LIMITED) 10 July 1991 (1991-07-10) *cf. page 3, lines 39-42, page 4,lines 29-30 and page 5, example 5* ---	1-28
X	WO 98 33494 A (KOSBAB JOHN V) 6 August 1998 (1998-08-06) *cf. abstract, page 3, lines 25-36* -----	1-11

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 01/00056

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-8
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1 - 28

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-8

Present claims 1-8 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds listed in the application.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-28

   PHARMACEUTICAL COMPOSITIONS COMPRISING ONE OR MORE FLAVONOIDS AND THEIR USE FOR THE TREATMENT OF BRAIN DISORDERS, INCLUDING NEURODEGENERATIVE DISORDERS, PARKINSON'S DISEASE AND BRAIN TUMORS.

2. Claims: 29-30, 33-36

   AN ASSAY METHOD FOR IDENTIFYING AN AGENT THAT CAN *DOWNREGULATE THE EXPRESSION OF MMP-2 AND/OR MMP-9.*

3. Claim : 32

   USE OF AN AGENT CAPABLE OF DOWNREGULATING EXPRESSION OF MMP-2 AND/OR MMP-9 IN THE PREPARATION OF A MEDICAMENT FOR THE TREATMENT OF BRAIN DISORDERS

INTERNATIONAL SEARCH REPORT

rmation on patent family members

Interr--'--al Application No
PC', 03 01/00056

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